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13. ABSTRACT (Maximum 200 Words) <p><i>Drosophila</i> Wingless (Wg) is the homologue of the vertebrate Wnt-1 that is implicated in breast cancer. I have investigated the role of heparan sulfate proteoglycans (HSPGs) in Wg signaling. HSPGs are cell surface macromolecules that consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. My genetic studies in <i>Drosophila</i> have uncovered critical functions of HSPGs in Wg signaling. First, I have shown that in the absence Sulfateless (Sfl), an essential enzyme involved in the biosynthesis of HS GAGs, Wg signaling is defective, suggesting that HSPGs play key role(s) in Wg signaling. I further demonstrated that <i>Division abnormally delayed</i> (Dally), a <i>Drosophila</i> HSPG of Glypican-type, is involved in Wg signaling. Genetic interaction experiments are consistent with a model in which Dally acts as a co-receptor for Wg. Finally, I have found that <i>Dally-like protein</i> (Dlp), a 2nd member of <i>Drosophila</i> Glypican play a key role in the regulation of the extracellular distribution of Wg protein. These findings provide new insights into our understanding of the extracellular regulation of Wnt signaling and will help for therapeutic interventions by the development of specific drugs for the prevention and treatment of breast cancer.</p>					
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Introduction

Wnt proteins are a large family of secreted, cysteine-rich glycoproteins that act as ligands to stimulate receptor-mediated signal transduction pathways in both vertebrates and invertebrates. Genetic studies in *Drosophila* and *Caenorhabditis elegans*, ectopic gene expression in *Xenopus*, and gene knockouts in mouse have demonstrated the involvement of Wnts in directing cell fates in many developmental processes including segmentation, CNS patterning, tissue induction, and asymmetric cell division (Wodarz and Nusse, 1998; Siegfried and Perrimon, 1994). Accumulating evidence has also demonstrated that inappropriate activation of the Wnt signal transduction pathway plays a role in a variety of human cancers including breast cancer (Peifer and Polakis, 2000; Polakis, 1999). The first member of this family, Wnt-1, was originally identified as a proto-oncogene in several mouse mammary tumors induced by mouse mammary tumor virus (Nusse and Varmus, 1982). Ectopic expression of Wnt-1 either in the mammary gland of transgenic mice (Thomas and Capecchi, 1990) or in cultured mammary epithelial cells (Brown et al., 1986; Rijsewijk et al., 1987) leads to morphological transformation, suggesting that Wnt-1 can have a profound effect on cell proliferation and differentiation. Importantly, mutations of genes that are normally involved in Wnt signaling were identified in human cancers. For example, mutations in β -catenin protein, a major component in Wnt signal transduction pathway, have been identified in both human tumors and cancer cell lines (Rubinfeld et al., 1997; Morin et al., 1997). These mutations lead to accumulation of β -catenin in the cells, a phenomenon that mimics the constitutive activation of Wnt signaling in cells. Further, the tumor suppressor adenomatous polyposis coli (APC) has also been demonstrated as a negative regulator in Wnt signal transduction pathway (Polakis, 1999). Altogether, these findings demonstrate that Wnt signaling may play an important role in the genesis and development of human cancers including breast cancer.

Drosophila genetics provides a great system to study Wnt function and its signal transduction pathway. The *Drosophila* *wg* encodes the homolog of vertebrate Wnt-1. Wg has been implicated in a number of developmental processes including embryonic segmentation, tissue induction and imaginal disc patterning (Wodarz and Nusse, 1998; Siegfried and Perrimon, 1994). *Drosophila* embryos are divided into segmentation units, which are determined by a hierarchy of maternal and zygotic genes. Each segment of the larvae is composed of alternating patterns of naked cuticle and cuticle covered with denticles. The specification of the naked cuticle is mediated in part by Wg. In *wg* mutant embryos, the naked cuticle is lost and replaced by a mirror image duplication of rows of denticles. Analyses of mutants that have cuticle similar to *wg* mutant constitute a very promising approach to identify molecules involved in Wg signal transduction pathway. Phenotypic analyses and genetic interaction studies in *Drosophila*, in combination with biochemical studies, have led to the identification of a number of downstream molecules required for Wg signaling (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Cox and Peifer, 1998). According to a current model, Wg initiates a signaling cascade by the Frizzled class of receptors. Activated Wg receptors lead to the activation of the modular protein Dishevelled. Activation of Dishevelled reduces the function of Zeste white 3 (Zw3) through binding components of the degradation complex consisting of APC, Axin and Armadillo (Arm). This results in the accumulation of cytoplasmic Arm. Arm subsequently translocates into the nucleus to form a complex with LEF/TCF transcription factors. Together Arm and TCF regulate the expression of many downstream target genes such as *engrailed* (*en*) in the embryonic epidermis, *distalless* (*dll*) and genes of the *achaete scute complex* (*ASC*) in the wing. Importantly, the Wg signal transduction pathway is conserved in both vertebrates and

invertebrates. For example, *Drosophila* Arm is the homolog of vertebrate β -catenin and plakoglobin. Thus, genetic studies of Wg signaling in *Drosophila* will provide new insights into mechanism(s) of both development and oncogenesis.

Despite the rapid progress in the understanding of intracellular events triggered by Wg/Wnt protein, it is not well understood how Wg signaling is regulated from the time of its synthesis to forming active complexes with its receptors. For example, Wg protein functions as a morphogen to pattern wing. It is not understood how Wg proteins move through tissues to establish the morphogen gradient. Furthermore, recent evidence suggests that both *Drosophila* Frizzled and Frizzled 2 function as two redundant Wg receptors. However, it is not clear about other component(s) involved in Wg/receptor complex. Thus, in regards to Wg signaling, two major important questions remain unresolved: **1. How do secreted Wg proteins form active complexes with their receptors; and 2. how do Wg proteins move through tissue.**

Heparan sulfate proteoglycans (HSPGs) are cell surface macromolecules that consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached (Bernfield et al., 1999). Glypicans and Syndecans represent the two major cell surface HSPGs. Syndecans are single membrane spanning proteins with extracellular domains that carry HS chains near their N-terminus. Glypicans are glycosyl phosphatidylinositol (GPI)-linked proteins bearing HS chains near their C-terminus. While Syndecans bear both HS and chondroitin sulfate (CS) GAGs, Glypicans are exclusively attached to HS GAGs. Through a series of modifications in HS GAG chains, enormous structural heterogeneity can be generated. Thus, both the modifications of HS GAG chains and the nature of the core proteins can potentially play roles in the specificity and function of individual HSPGs.

A large body of evidence, mainly from *in vitro* biochemical studies, has implicated HSPGs as important molecules in a variety of cellular functions such as cell adhesion, motility, proliferation, differentiation and morphogenesis (Bernfield et al., 1999, Iozzo, 1998). In the context of signal transduction, HSPGs have been proposed to act as co-receptors for a number of growth factors, internalization of receptors and transport of signaling molecules (Ornitz, 2000). The role of HSPGs in FGF signaling has been particularly well analyzed. In addition to its high affinity receptor, biochemical studies indicate that Heparin/HSPGs act as low affinity FGF co-receptors that facilitate FGF signal transduction (Schlessinger et al., 1995, Ornitz, 2000). However, the function of HSPGs *in vivo* has been unclear. Recent genetic studies in both *Drosophila* and mice have begun to uncover the functions of HSPGs *in vivo* in specific signaling mediated by multiple growth factors including Wg, Decapentaplegic (Dpp), Hedgehog (Hh) and FGFR (Perrimon and Bernfield, 2000).

The goal of this research fellowship is to investigate the role of HSPG in Wg/Wnt-1 signaling using *Drosophila* as a model system. Followings are the key research accomplishments that I made during the period of this research fellowship.

Key Research Accomplishments

Heparan Sulfate Proteoglycans play non-redundant roles with other classes of proteoglycans in the context of Wg signaling (Lin and Perrimon, 1999, See Appendices: paper 3).

Before I started this fellowship research, I and others have reported that in the absence of *sugarless* (Binari et al., 1998; Haecker et al., 1998; Haerry et al., 1998), Wingless (Wg) signaling

is defective. In mammalian cells this enzyme is required for the formation of Glucuronic acid (GlcA) (Hempel et al., 1994). Because GlcA is required for the formation of HS, chondroitin sulfate (CS) and dermatan sulfate (DS), the identity of the classes of proteoglycans involved in Wg signaling could not be determined. During the research period of this fellowship, I have characterized mutations in the gene *sulfateless* (*sfl*), which encodes a *Drosophila* homolog of vertebrate HS N-deacetylase/N-sulfotransferase (NDST). In *sfl* null embryos, expression patterns of *wg* and *en* are reminiscent of those observed in either *wg* or *hedgehog* (*hh*) null mutants and thus are consistent with a role for *sfl* in either Wg and/or Hh signaling. To further substantiate a requirement for Sfl activity in Wg signaling, we analyzed the effect of *sfl* mutations in the development of the stomatogastric nervous system (SNS) and the second midgut construction that require Wg activity. Examination of *sfl* null embryos revealed that in *sfl* null embryos, the development of these Wg mediated processes are perturbed. Consistent with a role for Sfl in Wg signaling, we found that Wg-dependent processes in the wing imaginal disc also require Sfl activity. Wg is required for D/V patterning and act short range to control the expression of *neuralized* (*neu*) at the wing margin and long range to activate the transcription of *distalless* (*dll*). In *sfl* mutant wing discs, the expression of *neu* is abolished, and the expression of *Dll* is also strikingly reduced. Altogether, our results indicate that Sfl activity is necessary for Wg signaling in both embryonic and wing disc development.

I have subsequently cloned the gene that encodes the product of *sfl*. *In vitro* transcribed *sfl* RNAs injected into marked *sfl* null embryos were able to rescue the *sfl* null embryonic phenotype suggesting that the cDNA we have isolated corresponds to *sfl*. Sfl encodes a *Drosophila* homolog of vertebrate HS N-deacetylase/N-sulfotransferase (NDST). In mammalian cells this enzyme is required specifically for the modification of HS GAGs but not CS and DS GAGs (Hashimoto et al., 1992) providing evidence that HSPGs are involved in Wg signaling and that HSPGs play non-redundant roles with other classes of proteoglycans in the context of Wg signaling.

Role of Dally in Wg signaling (Lin and Perrimon, 1999, see Appendices: paper 3)

Genetic analyses in *sgl* and *sfl* implicate a role of HS GAGs, but not other classes of GAGs in proper Wg signaling. Since HS GAGs are attached to various protein cores to form different HSPGs, I searched for candidate genes that could encode the protein core of the HSPG. I further demonstrated that Dally, a *Drosophila Glypican* is involved in the reception of Wg signaling (Lin and Perrimon, 1999; see Appendices). A number of genetic evidence supports this conclusion. First, hypomorphic *dally* alleles exhibit some wing margin defects (Nakato et al., 1995), a phenotype reminiscent of partial loss of *wg* activity. We find that the frequency of wing defect observed in *dally* mutant is increased by a reduction in *wg* or *sfl* activity suggesting that Dally plays a positive role in Wg signaling. Second, we used double-stranded RNA (dsRNA) interference as a method to abrogate the activity of the endogenous *dally* gene (Kennerdell and Carthew, 1998). We injected dsRNAs corresponding to the entire coding region of *dally* into wild type embryos. Embryos injected with the *dally* dsRNAs exhibit severe segment polarity cuticle defects, similar to those injected with *wg* dsRNAs (Kennerdell and Carthew, 1998). Finally, we conducted a number of genetic interaction experiments with members of the Wg signaling pathway. *dally* mutants can enhance the phenotype resulted from a loss of function *Drosophila Frizzled 2* (*Dfz2*), a receptor for Wg protein. Ectopic expression of a dominant negative form of *Dfz2* (*Dfz2N*), that encodes only the first extra-cellular domain and the first

transmembrane domain, has been shown to block Wg signaling, probably by binding to Wg in a non productive manner (Zhang and Carthew, 1998). When Dfz2N is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects. However, this phenotype is dramatically enhanced in homozygous *dally* mutants, suggesting that *dally* potentiates Wg signaling. Importantly, ectopic expression of a gain of function Arm protein (Arm s10) (Pai et al., 1997) can fully rescue the wing defects, suggesting that the enhanced wing margin defects by *dally* mutant is specific to Wg signaling and that Dally acts upstream of Arm. We also demonstrated that *dally* mutants can suppress the activities of Dfz2. Uniform overexpression of Dfz2 in the wing pouch leads to ectopic bristle formation in the wing blade, most likely reflecting the activation of Wg signaling above its normal level (Cadigan et al., 1998). In a *dally* mutant background, the formation of ectopic bristles was drastically reduced suggesting that a mutation in *dally* blocks the activity of Dfz2. Altogether, our genetic analyses as well as the results from others (Tsuda et al., 1999) are consistent with a role of Dally in Wg signaling and suggest that Dally may act together with Dfz2 in Wg reception (Lin and Perrimon, 1999, see Appendices).

HS GAGs are absolutely required for extracellular distribution of Wg (Baeg, Lin and Perrimon, 2000, See Appendix: paper 1).

Genetic studies have demonstrated that Wg can exert both short-range and long-range effects during the development of the embryo and imaginal discs (Bejsovec and Martinez Arias, 1991; Struhl and Basler, 1993). The role of Wg as a long-range morphogen has been clearly demonstrated in imaginal discs (Zecca et al., 1996; Neumann and Cohen, 1997). In wing imaginal discs, Wg is expressed in a narrow stripe of cells at the dorsal-ventral (D/V) compartment border and act long range. Wg acts up to 20 -30 cell diameters away from its site of synthesis and triggers a graded transcriptional response of target genes, such as *distalless(dll)*, which is a feature of morphogen molecules (Zecca et al., 1996; Neumann and Cohen, 1997). Recently, Strigini and Cohen (2000) were able to visualize the Wg extracellular protein gradient, thus providing direct support for this model. The transmembrane Wg receptor encoded by DFz2 (Bhanot et al., 1996) has been shown to play a critical role in shaping the distribution of Wg. DFz2 is required to post-transcriptionally stabilize Wg, and thus allows it to reach cells far from its site of synthesis (Cadigan et al., 1998). Our analyses of *sfl* strongly revealed a critical role of HS GAG in Wg protein signaling and distribution. When mutant clones null for *sfl* were induced, large notching phenotypes were observed, suggesting that HS GAGs are required for Wg signaling in wing patterning. To examine the role of HS GAG in Wg protein distribution, we generated *sfl* mutant clones in wing discs (Baeg, Lin and Perrimon, submitted, See Appendices). The distribution of Wg protein were examined by staining with anti-Wg antibody. As shown in Fig.6 (in Appendix: paper 1), when staining protocol that detects cytoplasmic Wg was used, we could not detect an alteration in the expression of intracellular Wg in *sfl* mutant clones, indicating cells mutant for *sfl* normally transcribe *wg* and do not accumulate Wg (Figure 6A,B in Appendix: paper 1). However, using the extracellular staining method, a dramatic decrease of extracellular Wg was detected in *sfl* mutant cells mutants (Figure 6C,D in Appendix: paper 1). These results strongly argue that HSPG(s) is critical for the distribution of extracellular Wg proteins (Baeg, Lin and Perrimon, see appendix paper 1).

***dally-like protein (dlp)* is a new segment polarity gene that may play an important role in extracellular distribution of Wg (Baeg, Lin and Perrimon, 2000, See Appendix: paper 1).**

We have recently identified and isolated a second member of *Drosophila* Glypican that was named as Dally-Like Protein (Dlp)(Baeg, Lin and Perrimon, submitted, See Appendices). Sequencing of the *dally* cDNA revealed a potential open reading frame of 769 amino acid residues that showed 22 and 35% identity to Dally and mouse K-glypican, respectively. The predicted primary structure of the molecule exhibits the hallmarks of a glypican protein. The hydrophilicity plot of the new molecule is similar to those of the other members of the family which is characterized by the presence of NH₂- and COOH-terminal hydrophobic signal sequences. Also, this molecule possesses a consensus serine/glycine dipeptide sequence for GAG attachment sites, and a signal sequence for a GPI-moiety attachment site at the COOH-terminal region. Further, the number and position of cysteine residues, which are a unique feature of glypican family members, are almost completely conserved in the predicted protein. These results suggest that Dlp is a novel *Drosophila* member of the glypican family. Hybridization using a *dlp* specific probe to polytene chromosomes from salivary glands localized the *dlp* gene to 70F on the third chromosome. Finally, Northern blot analysis revealed that *dlp* encodes a single major 3.8kb transcript.

To determine the function of DLP, we first determined the expression of *dlp* mRNAs in embryos by *in situ* hybridization. *Dlp* transcripts are uniformly expressed at early embryonic stages (Figure 2A in Appendix: paper 1), but by stage 8 are enriched in stripes (Figure 2B in Appendix: paper 1). Double staining for *dlp* mRNA and Wg protein show that *dlp* transcripts are preferentially expressed in three to four cells anterior to the *wg*-expressing cells (data not shown). Interestingly, this expression pattern is reminiscent to the expression of either *dally* or *DFz2* (Bhanot et al., 1996; Tsuda et al., 1999; Lin and Perrimon, 1999). Next, in an attempt to assess the function of DLP during embryogenesis, we used the RNA interference (RNAi) method (Kennerdell and Carthew, 1998) to perturb DLP protein synthesis. Embryos were injected with a *dlp* double-stranded RNA (dsRNA). These embryos showed a "segment polarity phenotype" which consists in the absence of naked cuticle (Figure 3D in Appendix: paper 1). This phenotype is reminiscent to loss of either *wg* or *hh* gene activities. Thus, we have identified a second member of *Drosophila* glypican *dlp* that acts as a segment polarity gene.

Our genetic analyses of *sfl* mutants implicate an important role of HSPG in the organization of the extracellular distribution of Wg proteins. Since Dlp is one of the major HSPG expressed in wing disc (X. Lin, unpublished results), we anticipated that Dlp might be a candidate of HSPG core that is involved in the regulation of extracellular distribution of Wg protein. If that is case, overexpression of Dlp would change the distribution of Wg protein in wing discs. Dlp was overexpressed in either A/P boundary or posterior regions of wing using Ptc gal4 or En-gal4 respectively. The effect of over-expressed DLP on Wg distribution was examined by staining these wing discs with anti-Wg monoclonal antibodies. We used two different staining protocols to detect Wg distribution. The first one involves fixation of the tissue before staining, and thus detects mostly cytoplasmic Wg present in either secretory or internalized vesicles (van den Heuvel et al., 1989; Neumann and Cohen, 1997). In the second protocol, the tissue is incubated with the antibody prior to fixation. Using the first protocol, in WT discs, Wg protein is found at high levels in a narrow stripe of three to five cells straddling the D/V boundary (Figure 5 in Appendix: paper 1). Using the second protocol, as previously observed by Strigini and Cohen (2000), extracellular Wg is organized in a gradient at the basolateral surface of *wg*-expressing and nearby cells. Following over-expression of DLP using

either *en-Gal4* or *ptc-Gal4*, a striking increased accumulation of Wg protein is observed (Figure 5 B,C in Appendix: paper 1). We also expressed Dally and *Drosophila* Syndecan in wing imaginal discs using *en-Gal4* or *ptc-Gal4*, we have not been able to detect a striking effects Wg protein distribution (X. Lin; unpublished observations). These results suggest that Dlp may have relative high capacity to bind Wg protein in comparison with Dally and *Drosophila* Syndecan.

Reportable Outcomes

Four papers listed in Appendices represented the major outcomes of this fellowship. Our findings reported in these papers illustrated an important progress in our understanding of the role of HSPG in Wg signaling in the context of developmental processes.

Conclusions

My genetic studies in *Drosophila* have uncovered critical functions of HSPGs in Wg signaling. First, I have shown that in the absence Sulfateless (Sfl), an essential enzyme involved in the biosynthesis of HS GAGs, Wg signaling is defective, suggesting that HSPGs play key role(s) in Wg signaling. I further demonstrated that *Division abnormally delayed* (Dally), a *Drosophila* HSPG of Glypican-type, is involved in Wg signaling. Genetic interaction experiments are consistent with a model in which Dally acts as a co-receptor for Wg. Finally, I have found that *Dally-like protein* (Dlp), a 2nd member of *Drosophila* Glypican play a key role in the regulation of the extracellular distribution of Wg protein. These findings provide new insights into our understanding of the extracellular regulation of Wnt signaling.

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Appendices:

1. Baeg, G., **Lin X.** (**First two authors have contributed equally to this work**) and Perrimon N. Heparan sulphate proteoglycans are critical for the organization of the extracellular distribution of wingless. Submitted.
2. **Lin X.** and Perrimon, N (2000) Role of heparan sulfate proteoglycans in cell-cell signaling in Drosophila. Matrix Biology 19, 303-307
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5. **Lin, X.**, Buff, E.M., Perrimon, N., and Michelson, A.M 1999 Heparan sulfate proteoglycans are essential for FGF receptor signaling during Drosophila embryonic development. Development, 126, 3715-23.

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Heparan Sulfate Proteoglycans are critical for the organization of the extracellular distribution of Wingless

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SUMMARY

Previously, we have shown that Heparan Sulfate Proteoglycans (HSPGs) are involved in Wingless (Wg/Wnt) signaling, and we identified the glypican gene *Dally* as encoding a putative protein core. Here, we report the identification of another *Drosophila* glypican gene, *dally-like protein* (*dlp*), and show that it is also involved in Wg signaling. Inhibition of *dlp* gene activity implicates a function for DLP in Wg reception, and we show that over-expression of DLP leads to an accumulation of extracellular Wg. We propose that DLP plays a role in the extracellular distribution of Wg. Consistent with this model, a dramatic decrease of extracellular Wg was detected in clones of cells that are deficient in proper glycosaminoglycan biosynthesis. We conclude that HSPGs play important role in organization of the extracellular distribution of Wg.

INTRODUCTION

Heparan sulfate proteoglycans (HSPGs) are abundant cell-surface molecules, which are part of the extracellular matrix. HSPGs consist of a protein core (such as syndecan and glypicans) to which heparan sulfate glycosaminoglycan (HS GAG) chains, which are long unbranched chains of sulfated repeating disaccharides, are attached (Kjellén and Lindahl, 1991; Lindahl et al., 1998). Numerous biochemical and cell-culture assays have suggested that HSPGs are critical for a variety of biological phenomena such as organogenesis, embryonic development, angiogenesis, regulation of blood coagulation, cell adhesion and lipid metabolism (Lindahl et al., 1994; Salmivirta et al., 1996; Rosenberg et al., 1997). In the context of signal transduction, HSPGs have been implicated in a number of signaling pathways, in particular Fibroblast growth factor (FGF), Wnt, Transforming growth factor (TGF)- β , and Hedgehog (Hh) (Reichsman et al., 1996; Lee et al., 1994; Rapraeger et al., 1991; Ruppert et al., 1996; The et al., 1999).

The function of HSPGs in signaling is well understood in the context of FGF signaling where they have been shown to stabilize the interaction between FGF and their transmembrane receptor

protein tyrosine kinases (FGFR). In the FGF context, the HSPG acts as a abundant low affinity receptors and recent structural studies have provided evidence that the HSPG plays a role in the interaction between FGFs and FGFRs (Plotnikov et al., 1999). These biochemical and structural findings are consistent with *in vivo* studies as well. In particular, genetic studies in *Drosophila* have identified mutations in two genes required for HS GAG biosynthesis; *sugarless* (*sgl*, UDP-glucose dehydrogenase; Häcker et al., 1996; Binari et al., 1996; Haerry et al., 1996) and *sulfateless* (*sfl*, *N*-deacetylase/*N*-sulphotransferase; Lin and Perrimon, 1999). Embryos that develop in the absence of either *sgl* or *sfl* gene activity are defective in FGF signaling (Lin et al., 1999).

Recently, evidence has been obtained in *Drosophila* for a role of HSPGs in the movement of the heparin-binding Hh proteins through tissues (Bellaiche et al., 1998; The et al., 1999). In the wing imaginal disc, Hh travels and acts at a distance of 8-10 cell diameters from the site of its production to induce the expression of its target gene *patched* (*ptc*) and *decapentaplegic* (*dpp*) along the anterior-posterior (A/P) boundary (Strigini and Cohen, 1997; Mullor et al., 1997). Results from mosaic analyses of *tout-velu* (*ttv*) mutations (Bellaiche et al., 1998), have revealed that *ttv* gene activity is required in Hh-receiving cells for the movement of Hh. Because, Ttv encodes a type II transmembrane HS polymerase enzyme, it has been proposed that a Ttv-modified HSPG is required for the proper distribution of the membrane-targeted cholesterol-modified Hh (HhNp) molecule through tissue (The et al., 1998). Recently, a novel Patched-like transmembrane protein, Dispatched (Disp), has been identified and shown to act exclusively in Hh-secreting cells to liberate HhNp from either the internal or surface membrane of the cells (Burke et al., 1999). One current model is that the Ttv-modified cell surface HSPG interacts with HhNp to release it from Disp, thus allowing HhNp to move through tissues (Burke et al., 1999; Ingham, 2000).

A number of evidence has also implicated a role for HSPGs in Wingless (Wg) signaling. In tissue culture cells, Wg proteins are tightly associated with cell membranes and the extracellular matrix, possibly through naturally occurring sulfated proteoglycans (Reichsman et al., 1996). Further, mutations in *sgl* and *sfl* are defective in Wg signaling (Häcker et al., 1996; Binari et al., 1996; Haerry et al., 1996; Lin and Perrimon, 1999). Recently, the gene *dally*, which encodes a *Drosophila* GPI (glycosyl-phosphatidyl inositol)-linked glypican molecule has been proposed to encode the protein

core of the HSPGs involved in Wg signaling (Lin and Perrimon, 1999; Tsuda et al., 1999). Loss of *dally* gene activity is associated with defects reminiscent of loss of *wg* activity, and genetic interaction between *dally* and known components of the Wg signaling pathway were found. These studies have led to the model that Dally plays a role in the reception of the Wg signal.

During patterning of the imaginal discs, Wg acts both as a short- and a long-range inducer (Zecca et al., 1996; Neumann and Cohen, 1997). Interestingly, in patterning the wing blade, Wg is expressed in a narrow stripe of cells at the dorsal-ventral (D/V) compartment border and act long range. Wg acts up to 20 -30 cell diameters away from its site of synthesis and triggers a graded transcriptional response of target genes, such as *distalless(dll)*, which is a feature of morphogen molecules (Zecca et al., 1996; Neumann and Cohen, 1997). Recently, Strigini and Cohen (2000) were able to visualize the Wg extracellular protein gradient, thus providing direct support for this model. The transmembrane Wg receptor encoded by DFz2 (Bhanot et al., 1996) has been shown to play a critical role in shaping the distribution of Wg. DFz2 is required to post-transcriptionally stabilize Wg, and thus allows it to reach cells far from its site of synthesis (Cadigan et al., 1998).

Two models have been proposed with regards to the function of HSPGs in Wg signaling (Reichsman et al., 1996). As observed in the case of FGF, HSPGs may be required for stabilizing a complex between Wg and its receptor. Alternatively, as in the case of heparin-binding growth factors such as vascular endothelial growth factor, heparin-binding epidermal growth factor (EGF) and TGF- β , the function of HSPGs may restrict the extracellular diffusion of the ligand.

Here we have examined the function of HSPGs in Wg signaling. First, we describe a novel glypican molecule in *Drosophila*, that we have named Dally-Like Protein (DLP), and show that it is required for Wg signaling. Interestingly, over-expression of DLP leads to an accumulation of extracellular Wg, suggesting that DLP plays a role in Wg extracellular distribution. Finally, we found that *sfl* mutant cells do not trap extracellular Wg proteins. Taken together, our results suggest that HSPGs act more indirectly in Wg signaling by modulating the concentration of the ligand.

RESULTS

Molecular cloning of a novel *Drosophila* glypican gene

Previously, one glypican molecule, Dally, was implicated in Wg signaling (Tsuda et al., 1999; Lin and Perrimon, 1999). However, because numerous *glypican* genes are present in other animals (Bernfield et al., 1999; Perrimon and Bernfield, 2000; Lander and Selleck, 2000), we searched the *Drosophila* database for additional *glypican* family members. We found a EST clone that showed some sequence similarity with Dally, and subsequently cloned a full length cDNA (see Materials and Methods). Sequencing of the cDNA revealed a potential open reading frame of 765 amino acid residues (Figure 1), that showed 22 and 35% identity to Dally and mouse K-glypican, respectively (Figure 1). The predicted primary structure of the molecule exhibits the hallmarks of a glypican protein. The hydrophilicity plot of the new molecule is similar to those of the other members of the family which is characterized by the presence of NH₂- and COOH-terminal hydrophobic signal sequences (Figure 1). Also, this molecule possesses consensus serine/glycine dipeptide sequences for GAG attachment sites, and a signal sequence for a GPI-moiety attachment site at the COOH-terminal region (Figure 1). Further, the number and position of cysteine residues, which are a unique feature of glypican family members, are almost completely conserved in the predicted protein (Figure 1). These results indicate that we have identified a novel *Drosophila* member of the glypican family, and we have named it Dally-Like Protein (DLP). Hybridization using a *dlp* specific probe to polytene chromosomes from salivary glands localized the *dlp* gene to 70F on the third chromosome (data not shown). Finally, Northern blot analysis revealed that *dlp* encodes a single major 3.8kb transcript (data not shown).

DLP is a novel segment polarity gene

To determine the function of DLP, we first determined the expression of *dlp* mRNAs in embryos by *in situ* hybridization. *dlp* transcripts are uniformly expressed at early embryonic stages (Figure 2A), but by stage 8 are enriched in stripes (Figure 2B). Double staining for *dlp* mRNA and Wg protein show that *dlp* transcripts are preferentially expressed in three to four cells anterior to the

wg-expressing cells (data not shown). Interestingly, this expression pattern is reminiscent to the expression of either *dally* or *DFz2* (Bhanot et al., 1996; Tsuda et al., 1999; Lin and Perrimon, 1999)

Next, in an attempt to assess the function of DLP during embryogenesis, we used the RNA interference (RNAi) method (Kennerdell and Carthew, 1998) to perturb DLP protein synthesis. Embryos were injected with a *dlp* double-stranded RNA (dsRNA) (see Materials and Methods). These embryos, referred to as *dlp* dsRNA embryos in the text, showed a “segment polarity phenotype” which consists in the absence of naked cuticle (Figure 2D). This phenotype is reminiscent of loss of either *wg* or *hh* gene activities. Similarly, the segment polarity phenotype is also found when the activity of *Dally*, which is required for Wg signaling in the embryo (Lin and Perrimon, 1999; Tsuda et al., 1999), was disrupted by *dally* RNA interference, though the effect is less severe than in *dlp* dsRNA embryos (Figure 2E). However, embryos injected with an equimolar mixture of *dlp* and *dally* dsRNAs showed more severe segment polarity phenotype compare with embryos injected with either *dlp* dsRNA or *dally* dsRNA alone, indicating that DLP and Dally may function redundantly in Wg signaling of embryonic stage (Figure 2F). Altogether, our results suggest that *dlp* is a novel segment polarity gene that potentiates Wg signaling.

Ectopic expression of DLP induces loss of Wg signaling

To further examine the role of DLP in Wg signaling, we analyzed the function of DLP during wing imaginal disc development. *dlp* transcripts are uniformly expressed in wing discs (data not shown). In the third instar imaginal disc, *wg* is expressed at the D/V compartment border and acts over short and long ranges to pattern the wing disc. Short range Wg signaling induces the expression of the proneural gene *achaete* (*ac*) in a stripe on each side of the D/V boundary, while long range Wg signaling controls the expression of *Dll* within the wing blade (Zecca et al., 1996; Neumann and Cohen, 1997). We reasoned that over-expression of DLP might activate Wg signaling because *dlp* dsRNA-injected embryos resemble those that have lost Wg activity. However, over expression of *dlp*, using the *C96-Gal4* driver, which is highly expressed at the D/V boundary of the wing disc, resulted in severe wing margin defects and loss of sensory bristles (Figure 3B). This phenotype is reminiscent of the phenotypes seen when Wg activity is reduced in the wing (Couso et al., 1994).

Consistent with the adult wing phenotype, Ac expression is dramatically decreased in wing discs over-expressing DLP (Figure 4B). Further, when *dlp* is over-expressed using the *engrailed-Gal4* (*en-Gal4*) driver, the expression of Dll is reduced in posterior compartment (Figure 4D).

Next, we tested whether over-expression of transducers of the Wg signal can rescue the loss-of-function *wg*-like phenotypes associated with *dlp* over-expression. Ectopic expression of either Wg or a gain-of-function Armadillo (Arm) can fully rescue the wing margin defects, and induced ectopic bristles that are characteristic of ectopic expression of the Wg pathway (Axelrod et al., 1996; Zhang and Carthew, 1998) (Figure 3C,D). Altogether, these results suggest that over-expression of DLP blocks Wg signaling in the wing disc, and that of DLP acts upstream of Arm.

Over-expression of DLP sequesters Wg

Since DLP is an extracellular GPI-linked molecule, we reasoned that patterning defects associated with DLP over-expression might reflect the ability of DLP to sequester Wg and thus prevent it to access and activate DFz2. To visualize the effect of over-expressed DLP on Wg distribution, we over-expressed DLP using various Gal-4 lines and stained the wing discs with anti-Wg monoclonal antibodies. We used two different staining protocols to detect Wg distribution. The first one involves fixation of the tissue before staining, and thus detects mostly cytoplasmic Wg present in either secretory or internalized vesicles (van den Heuvel et al., 1989; Neumann and Cohen, 1997). In the second protocol, the tissue is incubated with the antibody prior to fixation and mostly extracellular Wg can be visualized (Strigini and Cohen, 2000).

Using the first protocol, in WT discs, Wg protein is found at high levels in a narrow stripe of three to five cells straddling the D/V boundary (Figure 5A). Following over-expression of DLP using either *en-Gal4* or *ptc-Gal4* a striking increased accumulation of Wg protein is observed (Figure 5B,C). Using, the second protocol, as previously observed by Strigini and Cohen (2000), extracellular Wg is organized in a gradient at the basolateral surface of *wg*-expressing and nearby cells. Following over-expression of DLP, we detect an increased accumulation of extracellular Wg protein indicating that DLP affects extracellular Wg distribution (Figure 5D).

HSPGs are required to increase the local concentration of Wg ligand for its receptors

Our analysis of DLP, together with the previous findings on Dally (Tsuda et al., 1999; Lin and Perrimon, 1999), reveals that there are at least two HSPGs at the cell surface of wing disc cells that are involved in Wg signaling. To generate mutant cells that lack all GAGs and determine the role of the HSPGs in Wg signaling, we generated mutant clones of cells that do not properly synthesize GAGs. Previously, we have identified mutations in *sgl* and *sfl* (see Introduction) that are involved in the biosynthesis of GAGs. *Sgl* synthesizes Glucuronic acid that might freely diffuse between cells and acts cell non autonomously and thus cannot be used in this analysis (data not shown). On the other hand, *sfl* is involved in GAG modification and in its absence the GAG chains are not synthesized properly. For example, in *sfl* mutant third-instar larvae, heparin sulfate-modified Dally is significantly reduced and sharp bands of unmodified Dally are increased (Lin and Perrimon, 1999). Using the staining protocol that detects cytoplasmic Wg, we could not detect an alteration in the expression of intracellular Wg in *sfl* mutant clones, indicating cells mutant for *sfl* normally transcribe *wg* and do not accumulate Wg (Figure 6A,B). However, using the extracellular staining method, a dramatic decrease of extracellular Wg was detected in *sfl* mutant cells (Figure 6C,D). On the other hand, extracellular Wg was normally detected in cells outside the *sfl* mutant clones, indicating that secreted Wg freely moved across the clones and reached wild-type cells. Extracellular Wg has been shown to be mainly associated with the basolateral surface of cells, and GPI-anchored protein are thought to be primarily attached to the basal part of the cells. Together, these results reveal that the HSPGs are required for the binding of Wg to receiving cells.

Importantly, high accumulation of extracellular Wg can be detected on *sfl* mutant cells located next to the nearby wild-type cells (Figure 6D), suggesting that HSPGs act locally in a cell non autonomous manner. Consistent with the model, adult wing patterning in *sfl* mutant clones shows local cell non autonomy. Clones of *sfl* mutant cells are associated with wing margin defects suggesting that *sfl* is required for Wg signaling, however some of *sfl* mutant cells exhibit rescued *sfl* bristles (Figure 6E).

Taken together, our results suggest that in Wg signaling, HSPGs are involved in restricting Wg diffusion. Importantly, the local cell non autonomy observed in *sfl* mutant clones indicates that

HSPGs are not absolutely required for the binding of Wg to its receptors, as observed in the case of FGF (see below).

DISCUSSION

Wnt proteins are one of the first examples of secreted molecules that have been shown to be associated with both short- and long-range signaling activities during animal development (McMahon and Moon, 1989; Struhl and Basler et al., 1993). In the *Drosophila* wing imaginal disc, Wg is secreted from a narrow stripe, 3 to 5 cells wide along the D/V boundary and diffuse symmetrically in the extracellular space. Wg regulates the expression of different target genes in a concentration dependent manner (Zecca et al., 1996; Cadigan et al., 1998). Recently, Strigini and Cohen (2000) have shown that there is a basally-located gradient of extracellular Wg, which is most likely associated with extracellular matrix.

Here, we have identified and characterized a novel component of the Wg signal transduction pathway. We provide evidence that the putative GPI-attached Dally-Like Protein (DLP) potentiates Wg signaling pathway both during embryonic and imaginal disc development. Our results demonstrate that over-expression of DLP in wing discs sequesters Wg and disrupts its distribution. Finally, we provide direct *in vivo* evidence that HSPGs play a role in increasing the local concentration of extracellular Wg protein.

DLP encodes a HSPG core protein involved in Wg signaling

The predicted amino acid sequence of full-length DLP reveals that it is a new member of the glypican family of proteins (Figure 1). These proteins possess a C-terminal hydrophobic region that is thought to be required for processing and attachment to the external leaflet of the plasma membrane through a GPI linkage. Recent studies of Strigini and Cohen (2000) have clearly detected a broad gradient of extracellular Wg protein that is concentrated exclusively on the basolateral surface of *wg*-expressing and nearby cells. This distribution of Wg is consistent with an involvement of glypican proteins in Wg signaling because it has been reported that most of these molecules are located to the basolateral surface of polarized epithelial cells (Mertens et al., 1996).

We have obtained a number of evidence that implicate DLP in Wg signaling. First, *dlp* transcripts are expressed, as the Wg receptor *Dfz2*, at higher levels in segmentally repeated stripes anterior to the *wg*-expressing cells during segmentation (Figure 2B). Second, RNA-mediated interference of *dlp* supports the model that DLP potentiates Wg signaling, since *dlp* dsRNA embryos exhibit a *wg*-like segment polarity phenotype (Figure 2D). Third, double mutant embryos of *dlp* and *dally* from RNA interference showed genetic interaction between DLP and Dally (Figure 2F). Further, over-expression of DLP in the imaginal tissue generates a *wg*-like phenotype (Figure 3B) and is associated with trapping of extracellular Wg (Figure 5D). Finally, the phenotype associated with over-expression of DLP can be rescued by co-expression of a gain of function form of Arm (Figure 3D), which is consistent with the model that over-expressed DLP antagonizes Wg signaling.

Transducing Wg receptors and HSPGs

Morphogens are defined as localized factors that can diffuse and directly specify different cellular identities among a group of cells in a concentration-dependent manner (Wolpert, 1989). In the wing imaginal discs, Wg acts as a morphogen because it is organized in an extracellular protein gradient and activates the expression of target genes such as *ac*, *Dll* and *vestigial* (*vg*), in a dose-dependent manner (Zecca et al., 1996; Neumann and Cohen, 1997). In theory proteins that bind the morphogen molecules can play a role in shaping its extracellular distribution. Indeed, in the case of Wg, it has been shown *DFz2* is downregulated by Wg signaling. Thus, it has been proposed that a graded distribution of *DFz2* protein, which is opposite to the Wg gradient, exist in the wing disc (Cadigan et al., 1998).

Wg binds tightly to GAGs (Reichsman et al., 1996) and most likely binds DLP, raising the possibility that DLP is also involved in shaping the gradient of extracellular Wg. We have observed a high level of Wg accumulation in wing discs over-expressing *dlp*, suggesting that DLP may have a high capacity to bind Wg *in vivo*. Thus, the loss of Wg signaling activity that results from ectopic expression of DLP may simply reflect that Wg is trapped by the HSPG and is now unavailable for binding to *DFz2*. This model suggests that the amount of HSPG and *DFz2* transducing receptor may have to be precisely monitored to ensure proper patterning.

For simplicity in our discussion we have only taken DLP and DFz2 into account. However, the situation is more complex as there are at least two other putative Wg receptors, Fz1 and DFz3 (Sato et al., 1999), as well as one additional glypican molecule Dally (Lin and Perrimon, 1999; Tsuda et al., 1999), present at the surface of the wing disc cells. In the embryo Fz1 is able to substitute for DFz2 in transducing Wg (Kennerdell and Carthew, 1998; Chen and Struhl, 1999; Bhanot et al., 1999). DFz3 on the other hand acts as an antagonist of the pathway. Further, previous studies have shown a positive requirement for Dally in Wg signaling. With regards to Dally, we have not been able to detect a striking effect associated with its over-expression in the imaginal disc (data not shown). Possibly, this may reflect that Dally, unlike DLP, has a relatively low capacity to bind Wg *in vivo* (see also Strigini and Cohen, 2000). Future analyses of the mechanism of Wg signaling and distribution in the wing discs will have to take these other molecules into account. Further, it will also be important to monitor precisely the expression of each molecule in both WT and mutant contexts, as intricate regulatory loops exist. For example, *DFz3* is positively transcriptionally regulated, while both *Dally* and *DFz2* are negatively regulated by Wg signaling (Cadigan et al., 1998; Lin and Perrimon, unpublished).

The role of HSPGs in Wg signaling

To determine the role of HSPGs on Wg distribution, we generated clones of *sfl* mutant cells. In the absence of Sfl activity, presumably the GAG chains of all HSPGs are either not synthesized or modified properly.

The *sfl* mutant cells transcribe *wg* normally because we detected intracellular Wg protein as in the WT. Further, there is no effect on Wg secretion in these cells since we could not detect Wg accumulation, as observed in *porcupine* or *shibire* mutant clones (Strigini and Cohen, 2000). However, we could detect a dramatic decrease of extracellular Wg protein in *sfl* mutant clones suggesting that the trapping of Wg to *sfl* mutant cells is impaired.

Our mosaic analysis of *sfl* reveals a local cell non autonomy of the HSPG (Figure 6D,F), suggesting that the HSPG may not be important for activation of signaling receptors as previously described for the role of those molecules in FGF signaling that FGF is unable to induce FGF receptor

dimerization and activation in the absence of either free heparin sulfate or cell surface HSPG (Mason, 1994; Schlessinger et al., 1995). This result is consistent with our previous observations that overexpression of Wg protein can bypass the requirement of HS GAG in Wg signaling (Häcker et al., 1996). Altogether, our findings are consistent with the model that the function of the HSPG in Wg signaling is to limit Wg extracellular diffusion. Thus when Dlp is over-expressed, Wg is trapped and can not be presented to its transducing receptors. One possible model is that in the WT, the abundant GAG chains of HSPG are required for reducing dimensionality for ligands, which in turn allow more frequent encounters with the high affinity signaling receptor, which are in low abundance. The local non-autonomy associated with *sfl* mutant clones could then be explained by the local diffusion of Wg from the surface HSPG present on nearby WT cells. Taken together, our results suggest that HSPGs act more indirectly in Wg signaling by limiting Wg protein diffusion and increasing the local concentration of Wg.

MATERIALS AND METHODS

Molecular Cloning of *dlp*

A 0- to 4- hour embryonic cDNA library (Brown and Kafatos, 1988) was screened using a ³²P-labeled 0.7Kb BamHI fragment from the EST clone CK00242 (Kopczynski et al., 1998) as a probe. A positive clone carrying the entire coding region was subcloned into pBluescript II KS (pBS(KS)-*dlp*, HindIII-EcoRI) and sequenced using synthetic oligonucleotides primers.

UAS Constructs and Ectopic Expression

UAS-dlp^{WT} was created by cloning the full-length (XhoI-XbaI) *dlp* fragment from pBS (KS)-*dlp* into pUAST. The construct was introduced into *w¹¹¹⁸* host by P-mediated germline transformation (Rubin and Spradling, 1982). Targeted ectopic expression was accomplished using the UAS/Gal4 system (Brand and Perrimon, 1993).

The Gal4 drivers and UAS lines used in this study are: *UAS-wg* (Binari et al., 1997), *UAS-arm^{act}* (Pai et al., 1997), *C96-Gal4* (Gustafson and Boulianne, 1996), *en-Gal4* on second chromosome and *ptc-Gal4* on third chromosome.

***In situ* hybridization and RNA-mediated interference**

dlp mRNA was detected in whole-mount embryos using digoxigenin-UTP-labeled RNA probes prepared from the pBS(KS)-*dlp*.

The CK00242 plasmid containing a 1.2Kb fragment of the C-terminus *dlp* coding sequence and pBS(KS)-Dally containing a 1.1Kb EcoRI-BamHI fragment from LP 11764, which includes the entire coding region of Dally, were linearized with the appropriate restriction enzymes, and transcribed *in vitro* with Ambion T3 and T7 Megascript Kits following the manufacturers instructions. Transcripts were annealed in TE buffer (10mMTris-HCl, 8.0 and 1mM EDTA, 8.0) after heating to 100°C for 1 min and cooling to room temperature overnight. Annealed transcripts were analyzed on 1% agarose gels to confirm the size of the annealed dsRNA. Wild-Type embryos were injected into precellular embryos as described by Kennerdell and Carthew (1998) with dsRNA: either 3μM *dlp* dsRNA, 3μM *dally* dsRNA or 1.5μM each of *dlp* and *dally* dsRNAs. Embryos were incubated at 18°C under oil for two days. For cuticle preparation, embryos were mounted in Hoyer's medium/lactic acid.

Antibody labeling

Cytoplasmic Wg proteins were detected as described by Cadigan and Nusse (1996). For labeling extracellular Wg proteins, third instar larvae were dissected in ice-cold Schneider's M3 Medium (Sigma) and incubated with mouse anti-Wg antibody (1:3) for 1 hr on ice. After washing 3 times with cold PBS, larvae were fixed in PBS containing 4% formaldehyde at room temperature for 20 min. Larvae were rinsed three times with PBS for 40 min and incubated with fluorescent secondary antibody overnight (Strigini and Cohen, 2000). Other antibodies used are: mouse anti-Dll (1:10) and mouse anti-Ac (1:10).

Loss-of-function Clonal Analysis

To study the role of HSPGs for extracellular distribution of Wg, *y w hsflp; ubiquitin-GFP FRT2A/TM6C* females were crossed with *sfl^{l(3)03844} FRT2A/TM6C* Wing discs from Non-Tubby larvae were dissected and stained. For the generation of adult *sfl* somatic clones in the wing, females of *y w hsflp122; sfl^{l(3)03844} FRT2A/TM3, Sb* were crossed with males of *y w; P[y⁺] FRT2A/TM3, Sb*.

Larvae from this cross were heat-shocked for 2 hr at first or second instar. Adult wings were mounted in Euparal for observation.

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Figure legends

Figure 1. Amino acid sequence of DLP.

The entire predicted amino sequence of DLP is aligned with Dally and mouse K-Glypican. DLP is 22% and 35% identical to Dally and mouse K-Glypican, respectively. Underlines indicate the hydrophobic stretches for the predicted signal sequences involved in secretion (amino acid residues 23-41), and GPI-anchoring (residues 744-765). The predicted GPI-anchor attachment sites are indicated by bold underline. The position of cysteine residues in glypican family members and serine-glycine dipeptide sequences in Dlp are boxed.

Figure 2. DLP is a novel segment polarity gene involved in Wg signaling.

In situ hybridization of WT embryos using a *dlp*-specific probe reveals that *dlp* RNA are uniformly expressed at stage 2 (A), and are enriched in a segmentally repeated pattern at stage 8 (B). Embryos injected with a *dlp* dsRNA show a segment polarity phenotype characterized by the absence of naked cuticle (D, compare with the embryos injected with buffer in C). Similarly, embryos injected with *dally* dsRNA develop *wg*-like cuticle defects (E). On the other hand, embryos injected with equimolar of mixture of *dlp* and *dally* dsRNAs exhibit more severe segment polarity phenotype reminiscent of *wg* or *hh* null mutations (F).

Figure 3. Ectopic expression of DLP induces a *wg*-like phenotype.

Ectopic expression of *dlp* using the *C96-Gal4* driver generates wing margin defects and loss of sensory bristles. (B) is a *C96-Gal4 / UAS-dlp^{WT}* wing; compare with the WT shown in (A). Co-expression of *wg* (C, *UAS-wg/+; C96-Gal4,UAS- dlp^{WT}/+*), or a gain of function Arm (D; *C96-Gal4,UAS- dlp^{WT}/UAS-arm^{act}*) are sufficient to fully rescue both the wing notching phenotype and the loss of sensory bristles associated with over-expression of DLP. In addition, ectopic margin bristles close to the wing margin are observed.

Figure 4. Effect of over-expressed DLP on Wg-short and -long range target genes.

Ac is expressed in two parallel bands at the presumptive anterior wing margin (A), and is a short range target gene of Wg (Zecca et al., 1996; Neumann and Cohen, 1997b). In a *C96-Gal4 / UAS- dlp^{WT}* wing disc, *ac* expression is dramatically decreased (B). In the wing blade, *Dll*, a long-range target gene of Wg and is normally expressed in a wide domain with its highest level at D/V boundary (C). In a *en-Gal4/+; UAS-dlp^{WT}/+* discs, the expression of *Dll* is abolished in the posterior compartment (compare to the expression in the anterior compartment in D).

Figure 5. Over-expressed DLP sequesters extracellular Wg.

Wg (in green) is detected in a narrow stripe of 3 to 4 cells straddling the D/V boundary of the wing disc using a conventional(con) staining protocol that detect mostly cytoplasmic Wg (A-C; see Results). (A). Ectopic expression of DLP-WT, using *en-Gal4* and *ptc-Gal4*, results in an increased accumulation of Wg. (B) shows the trapping of Wg in the posterior compartment in an *en-Gal4 /+; UAS-dlp^{WT} /+* disc, and (C) shows the trapping of Wg at the anterior/posterior (A/P) boundary in a *ptc-Gal4 / UAS-dlp^{WT}* disc. (D) Imaginal discs were incubated with anti-Wg antibody prior to fixation to visualize extracellular(ex) Wg. Over-expression of DLP caused accumulation of extracellular Wg (Gray) to high level in the entire posterior compartment.

Figure 6. HSPGs restrict Wg diffusion.

Cytoplasmic (A, B) and extracellular Wg protein (C, D) were visualized using different staining methods. Wg expression is shown separately (A, C). *sfl* mutant clones were detected by the absence

of GFP (B, D; see Experimental Procedures). No alteration in the expression of intracellular Wg proteins (Gray, Red) in *sfl* mutant clone was observed suggesting that *sfl* cells transcribe and secrete Wg normally (A, B). A dramatic decrease of extracellular Wg (Gray, Red) was detected in *sfl* mutant cells (C, D). Clones of *sfl* mutant in adult wing lead to nicks of wing margin, however some of *sfl* mutant cells marked with *yellow* (arrows) exhibit wild-type (E). The presence of rescued *yellow* bristles suggests that *sfl* mutant cells act local cell non autonomously.

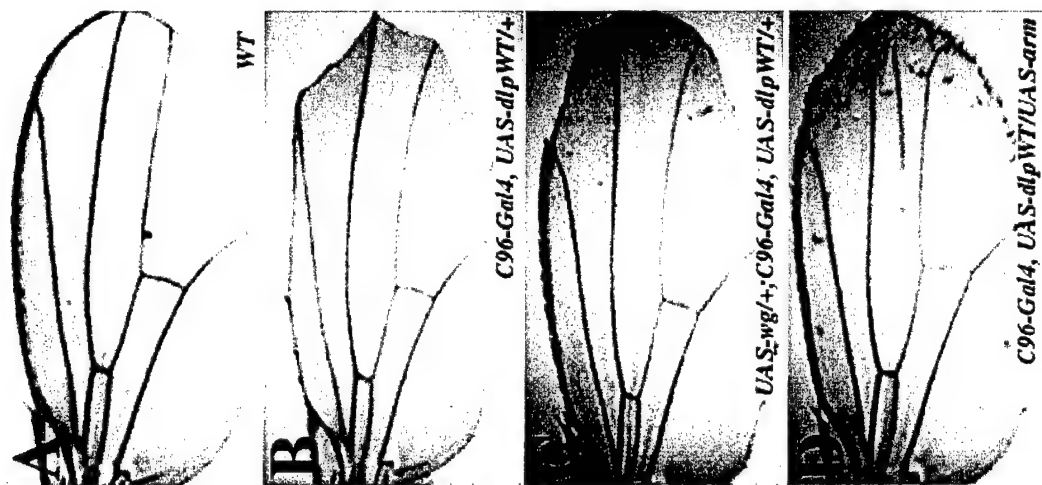


Fig. 3

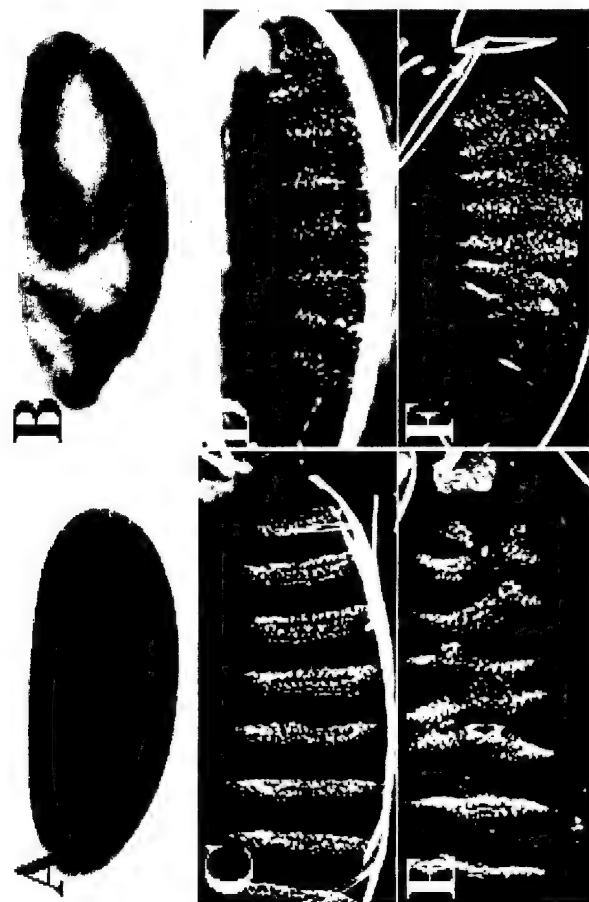


Fig. 2

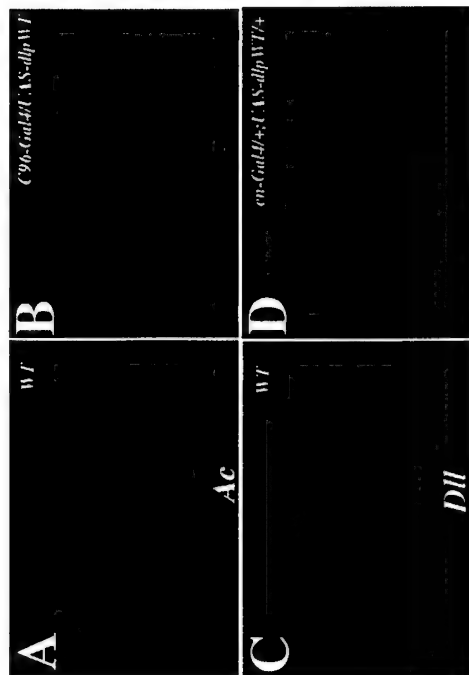


Fig. 4

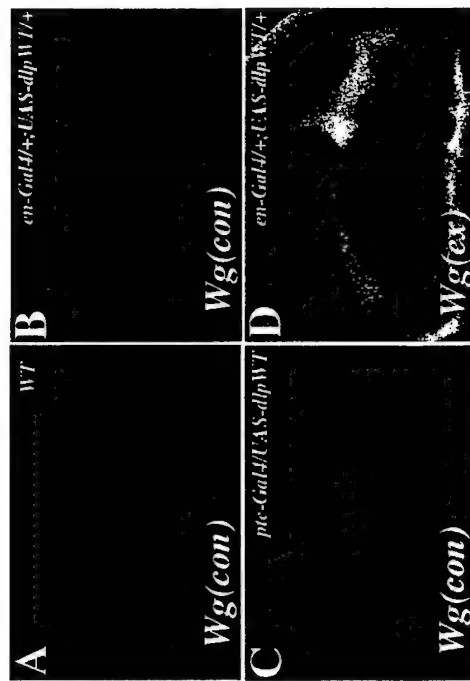


Fig. 5

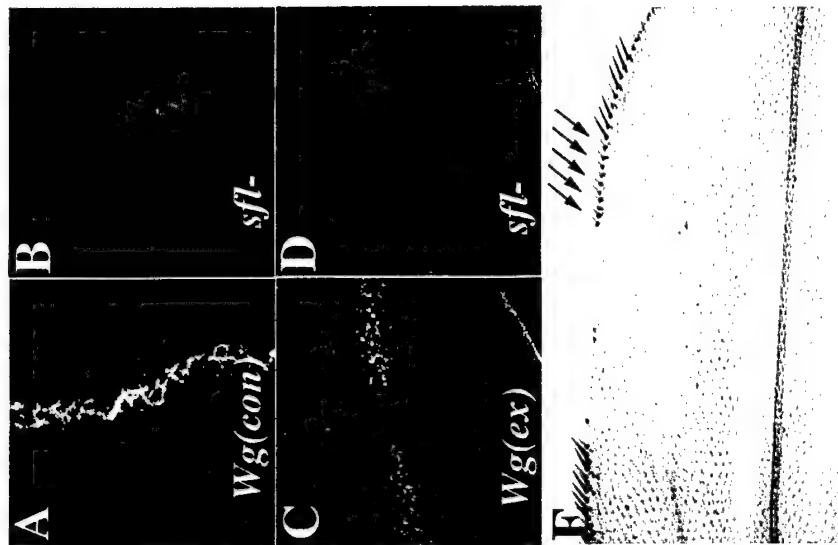


Fig. 6

Dally cooperates with *Drosophila* Frizzled 2 to transduce Wingless signalling

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The *Drosophila* wingless gene (*wg*) encodes a protein of the Wnt family and is a critical regulator in many developmental processes¹. Biochemical studies have indicated that heparan sulphate proteoglycans, consisting of a protein core to which heparan sulphate glycosaminoglycans are attached², are important for Wg function³. Here we show that, consistent with these findings, the *Drosophila* gene *sulfateless* (*sfl*), which encodes a homologue of vertebrate heparan sulphate *N*-deacetylase/*N*-sulphotransferase (an enzyme needed for the modification of heparan sulphate) is essential for Wg signalling. We have identified the product of *division abnormally delayed* (*dally*), a glycosyl-phosphatidyl inositol (GPI)-linked glypican, as a heparan sulphate proteoglycan molecule involved in Wg signalling. Our results indicate that Dally may act as a co-receptor for Wg, and that Dally, together with *Drosophila* Frizzled 2, modulates both short- and long-range activities of Wg.

Wg signalling is defective in *sugarless* (*sgl*) mutants⁴. *sgl* encodes a *Drosophila* homologue of uridine diphosphoglucose dehydrogenase that is required for the formation of glucuronic acid. Because glucuronic acid is required for the formation of heparan sulphate, chondroitin sulphate and dermatan sulphate, it is unclear which classes of proteoglycans are involved in Wg signalling. In the genetic screen⁵ that led to the isolation of *sgl*, we also isolated mutations in a second locus *sulfateless* (*sfl*), that showed a similar segment-polarity cuticle phenotype (Fig. 1b). In *sfl* null embryos, the expression patterns of *wg* and *engrailed* (*en*) are reminiscent of those observed in either *wg* or *hedgehog* (*hh*) null mutants (not shown). To investigate further whether Sfl activity is required in Wg signalling, we analysed the effect of *sfl* mutations on the development of stomatogastric nervous system (SNS) and the second midgut

constriction, both of which require Wg but not Hh activity⁶. In *sfl* null embryos, the development of these Wg-mediated processes is perturbed (Fig. 1e, g). Consistent with a role for Sfl in Wg signalling, Wg-dependent processes in the wing imaginal disc also require Sfl activity. Wg is required for dorso-ventral patterning and acts over a short range to control the expression of *neuralized* (*neu*) at the wing margin¹ and in the long range to activate the expression of *distalless* (*dll*)^{7,8}. In *sfl*-mutant wing discs, the expression of *neu* is abolished (Fig. 1i), and *Dll* expression is also markedly reduced (Fig. 1k). Our results indicate that Sfl activity is necessary for Wg signalling during both embryonic and wing-disc development.

A complementary DNA encoding the product of *sfl* was isolated (Fig. 2). A search of the protein sequence databases revealed that the putative protein deduced from the *sfl* cDNA is homologous with heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST)⁹, which is required specifically for the modification of heparan sulphate glycosaminoglycans (GAGs) but not chondroitin sulphate and dermatan sulphate GAGs. Together, these results provide genetic evidence that heparan sulphate proteoglycans (HSPGs) are involved in Wg signalling and that HSPGs have non-redundant roles with other classes of proteoglycan in the context of Wg signalling.

Heparan sulphate GAGs are attached to various protein cores to form different HSPGs. Because *Drosophila* frizzled 2 (*Dfz2*) appears as a distinct band on western blots (S. Cumberledge, personal communication; M. Zeidler and N.P., unpublished), not as the smear that is characteristic of proteoglycans², it is unlikely that the receptors for Wg/Wnt encoded by members of the Frizzled (*Fz*) family are heparan-sulphate-modified proteins. The *Drosophila* glypican homologue *dally* appeared to be an excellent candidate because flies homozygous for hypomorphic *dally* alleles exhibit some wing-margin defects¹⁰, a phenotype similar to partial loss of *wg* activity. To examine the role of *dally* in Wg signalling, we first determined the expression of *dally* messenger RNAs in embryos by *in situ* hybridization (Fig. 3). At early stages *dally* transcripts are uniformly expressed; however, at stage 8, *dally* transcripts are enriched in a segmental repeated pattern in three to four cells anterior to *wg*-expressing cells. Double staining for *Dfz2* mRNA and *wg*-lacZ shows that the 2–3-cell-wide band of *dally*-expressing cells anterior to *wg*-expressing cells also express *Dfz2*¹¹ (not shown), indicating that *dally* may be involved in Wg signalling.

Next, we examined the cuticle phenotype of *dally* mutant

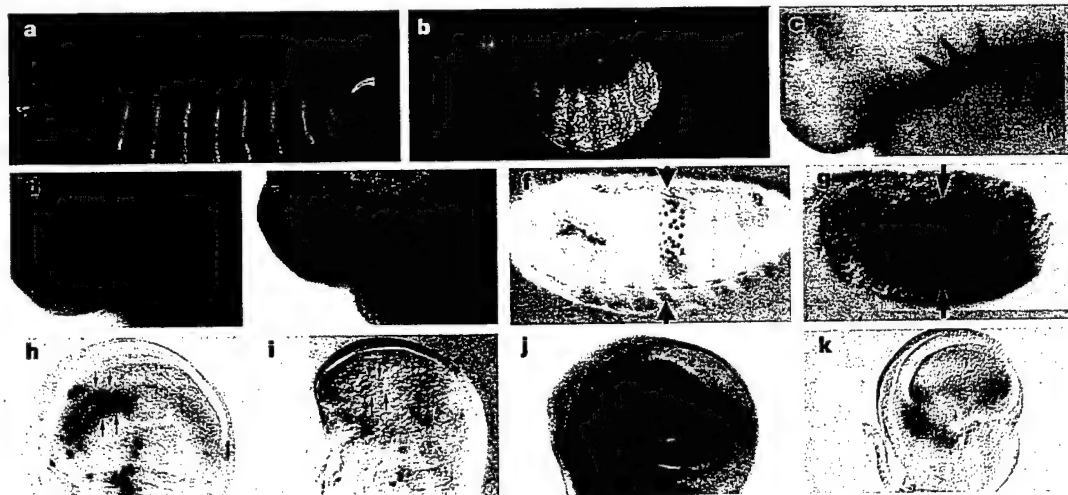


Figure 1 *sfl* is required for Wg signalling. **a, b**, Cuticle phenotypes of wild-type (WT) (**a**) and *sfl* (**b**) embryos. **c–e**, SNS phenotypes stained by anti-Crumbs antibody in stage 10 embryos of WT (**c**), *wg* (**d**), *sfl* (**e**). As observed for *sgl*⁶, the SNS phenotype is similar, although slightly weaker, than in the *wg* mutant (ref. 6). **f, g**, The expression of Labial (*Lab*) in WT (**f**) and *sfl* (**g**) embryos at stage 15; *Lab* staining, marking the position where the second midgut (arrows) is absent in an

sfl embryo (**g**). In the wing disc of WT third-instar larvae (**h**), *neuralized* (*neu*)-expressing sensory mother cells visualized using the A101 enhancer trap are found in two rows (arrows), and are missing in *sfl*⁽³⁾⁰³⁸⁴⁴/*sfl*⁹⁸⁴ wing disc derived from *sfl* homozygous mutant animals derived from heterozygous mothers (**i**). In WT wing disc (**j**), *Dll* forms a gradient with its highest level of expression at the dorso-ventral boundary, and is greatly reduced in the *sfl*⁽³⁾⁰³⁸⁴⁴/*sfl*⁹⁸⁴ disc (**k**).

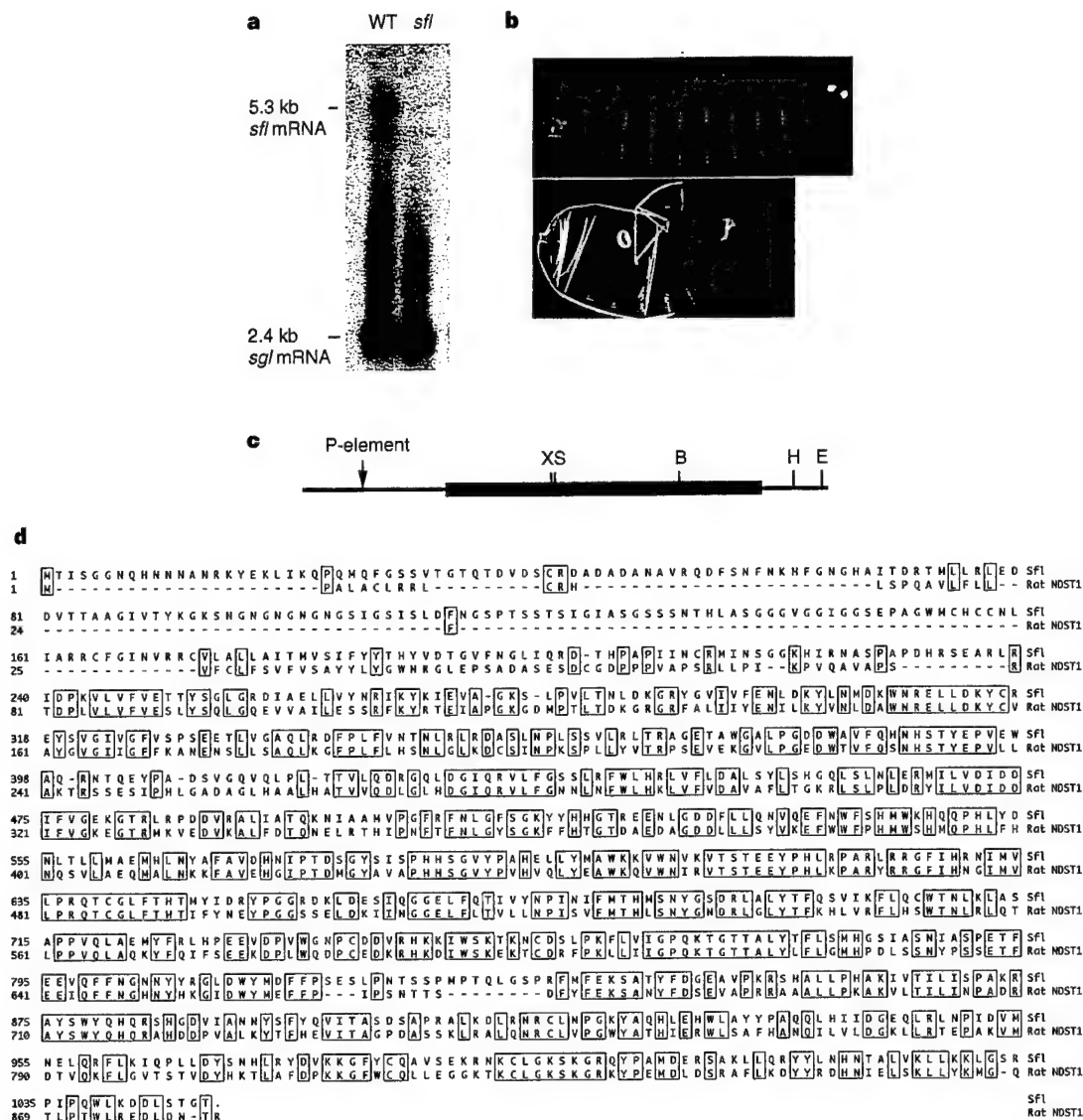


Figure 2 *sfl* encodes heparan sulphate *N*-deacetylase/*N*-sulphotransferase (NDST). **a**, Northern blot analysis of *sfl* RNA from 0–1.5 h WT and *sfl* mutant embryos. The blot was probed with *sfl* cDNA and *sgl* cDNA⁶. *sgl* was used as an internal control. 5.3 and 2.4 kilobase (kb) mRNAs correspond to the *sfl* and *sgl* transcripts, respectively. **b**, Rescue of *sfl* maternal-effect phenotypes by RNA injection. Top, cuticle phenotype of a paternally rescued *sfl* embryo marked with a *trachealess* (*trh*) mutation that exhibits the defective posterior spiracles⁶. Bottom, cuticle phenotype of *sfl* null embryos derived from GLCs injected with RNA transcribed from the *sfl* full-length cDNA. Of 700 injected embryos derived from

females with *trh* GLCs, 120 *sfl* mutant embryos (*trh sfl/sfl*) developed scorable cuticle structures, and 15% of them showed evidence of rescue. **c**, Restriction map of the *sfl* cDNA. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sst*I; X, *Xho*I. The P-element *l*(3)03844 is inserted at base 576 of the *sfl* cDNA (686 base pairs (bp) upstream of a putative ATG start codon). The open reading frame encoding Sfl is shown by the thick line. **d**, Putative amino-acid sequence of Sfl protein and comparison with rat NDST1. Identical residues are boxed. The overall identity between Sfl and Rat NDST1 is 51%.

embryos. These mutant embryos exhibit poorly penetrant cuticle segment-polarity defects resembling a partial defect in Wg signalling (Fig. 3d). However, these segmentation defects can be significantly enhanced by removal of one copy of *sfl* in the mother or one copy of *wg* in the embryo (Fig. 3e, f). Because all available *dally* mutations are weak alleles, we used double-stranded RNA (dsRNA) interference to block *dally* gene activity¹². Embryos injected with *dally* dsRNAs corresponding to the entire coding region of *dally* exhibit severe segment-polarity defects (Fig. 3g, h), similar to those injected with *wg* or *frizzled* (*fz*) + *Dfz2* dsRNAs¹². This result, together with the genetic interaction observed between *sfl* and *wg*, strongly supports the proposal that *dally* is a new segment-polarity gene and that it is required for Wg signalling in the embryo. Further, we find that Dally, which migrates as a smear in wild-type extracts, migrates as sharp bands in the protein extracts of *sfl* mutants (Fig. 4). Similarly, Dally is not modified in *sgl* embryos (S. Selleck, personal

communication). These results indicate that Dally is a likely substrate of Sgl and Sfl.

To further examine the role of *dally* in Wg signalling, we analysed the function of *dally* during wing-disc development. Consistent with previous reports¹⁰, we found that only 3% of homozygous *dally* animals exhibit wing-margin defects (Fig. 5a). This frequency can be increased 2–3-fold and wing-margin defects are more severe when one copy of *wg* is removed (Fig. 5b). To determine whether Dally cooperates with the Wg receptor Dfz2 in wing patterning, we tested whether *dally* mutations can enhance a loss-of-function *Dfz2* phenotype. When a dominant-negative form of *Dfz2* (*Dfz2N*)¹³ is expressed ectopically using the Gal4 line C96, which drives expression in the presumptive wing margin, flies develop partial margin defects (Fig. 5c). However, this phenotype is enhanced in homozygous *dally* mutants (Fig. 5d), indicating that *dally* may potentiate Wg signalling. Furthermore, ectopic expression of a gain-of-function

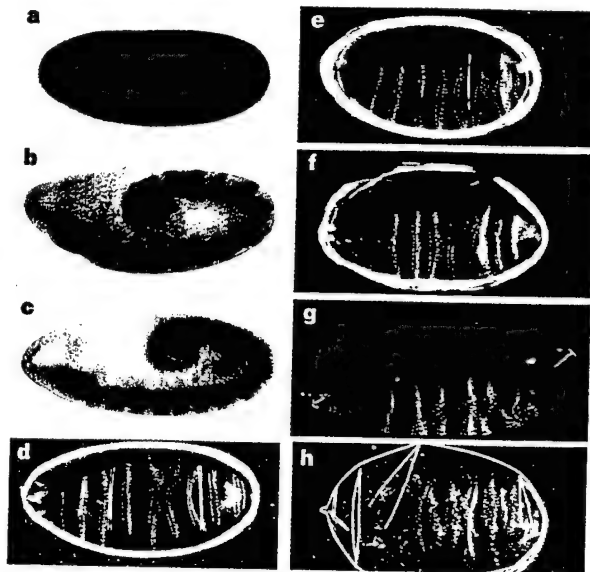


Figure 3 *dally* is a segment-polarity gene. *dally* mRNAs are uniformly distributed in stage 2 embryos (a), and are expressed in a segmental repeated pattern at stage 8 (b). c, *dally* transcripts (blue) are located 3–4 cells anterior to *wg*-expressing cells (brown, *wg-lacZ*). *dally*^{P2} homozygous embryos derived from females with *dally*^{P2} or *dally*^{ΔP-188} homozygous GLCs exhibit weak segment-polarity cuticle defects (d; 8% penetrance, *n* = 760). More severe defects are detected in *dally*^{P2} embryos where *sfl* maternal message is eliminated (e; 14% penetrance; *n* = 780). Similarly, *dally*^{P2} homozygous embryos derived from GLCs show a more severe embryonic phenotype if zygotic *wg* is reduced by half (f; 16% penetrance, *n* = 760). Embryos injected with *dally* dsRNA develop *wg*-like cuticle defects (g, h). 48% of the injected embryos (*n* = 127) exhibit defects. Embryos injected with buffer exhibit no cuticle defects (*n* = 150).

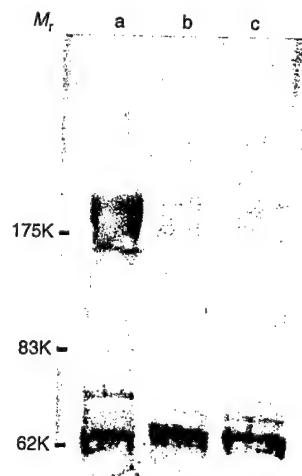


Figure 4 Heparan sulphate GAG modification of Dally in *sfl* mutants. Total proteins from third-instar larvae were analysed by SDS-PAGE followed by western blotting with anti-Dally antibody. In WT larvae (a), Dally migrates as a high relative molecular mass (*M_r*) smear, characteristic of heparan sulphate-modified Dally, and ~70K unmodified bands. In homozygous *sfl*⁽³⁰³⁰⁴⁴⁾ (b) or *sfl*⁽⁹⁸⁴⁾ (c) larvae, high *M_r* heparan sulphate-modified Dally is significantly reduced and sharp bands of unmodified Dally are increased.

Arm protein (*Arm*^{act}) can fully rescue the wing defects (Fig. 5f), indicating that the enhancement of wing-margin defects in the *dally* mutant is specific to Wg signalling and that Dally acts upstream of Arm. These genetic interactions are consistent with a role for Dally in Wg signalling and indicate that Dally may act with Dfz2 in Wg reception.

If *dally* acts with Dfz2 to transduce Wg signalling, Dally may also

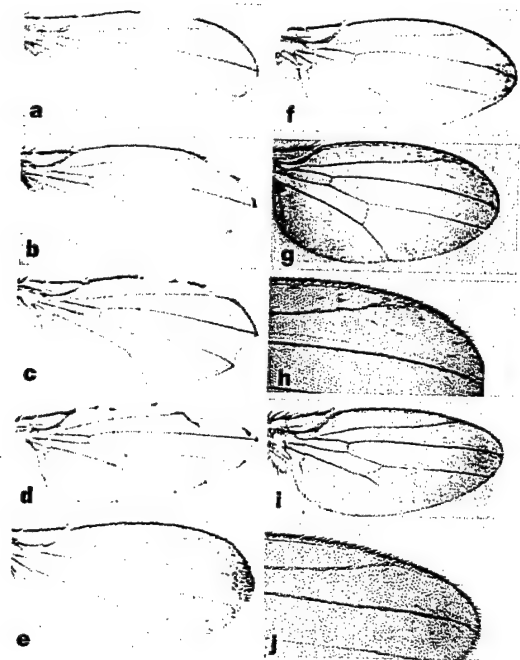


Figure 5 Dally is required for Wg/Dfz2 signalling in wing patterning. a, 3% of *dally*^{P2} homozygous flies exhibit a wing notching phenotype (*n* = 550). b, Wing phenotype of *wg*⁽³²²⁾ / +; *dally*^{P2} / *dally*^{P2} animals is enhanced and shows higher penetrance (8%; *n* = 654). The wing-vein abnormality seen in the *dally* mutant does not seem to involve Wg signalling. c, Ectopic expression of Dfz2N at the presumptive wing margin using C96-Gal4 (*UAS-Dfz2N* / +; C96 / +) is associated with a fully penetrant mild wing-margin defect. d, *UAS-Dfz2N* / +; C96 *dally*^{P2} / *dally*^{P2} wing. Decreased *dally* activity strongly enhances the wing defect observed in c. e, *UAS-arm*^{act} / +; C96 / + wing. Ectopic expression of *arm*^{act} results in ectopic bristles on the wing blade. f, *UAS-arm*^{act} / *UAS-Dfz2N*; C96 *dally*^{P2} / *dally*^{P2} wing. Ectopic expression of *UAS-arm*^{act} fully rescues the margin defect shown in d. g, h, Uniform expression of Dfz2 driven by 69B-Gal4 in 69B-Gal4 / *UAS-Dfz2* flies leads to wings with ectopic bristles. i, j, Ectopic bristles are strikingly reduced in the wing of *UAS-Dfz2* *dally*^{P2} / 69BGal4 *dally*^{P2}. h, j, Higher magnifications of the wings shown in g and i, respectively.

be required for other functions of Dfz2 in Wg signalling. In the wing blade, Dfz2 is involved in shaping the gradient of Wg distribution and determining the response of cells to Wg¹⁴. Uniform overexpression of Dfz2 in the wing pouch leads to ectopic bristle formation in the wing blade, probably reflecting activation of Wg signalling above its normal level. Ectopic expression of Dfz2 driven by the Gal4 line 69B resulted in wings with ectopic bristles¹⁴ (Fig. 5g, h). In a *dally* mutant background, the formation of ectopic bristles was greatly reduced, indicating that a mutation in *dally* blocks the activity of Dfz2 (Fig. 5i, j).

Our findings indicate that HSPGs have non-redundant roles with other classes of proteoglycan in Wg signalling, and that *dally* encodes a protein core of the HSPGs involved in Wg signalling. There are several possible mechanisms for the function of Dally in Wg signalling. First, Dally could form an active Wg receptor complex with Dfz2. Second, Dally, through its heparan sulphate GAG sequences, could generate a higher-affinity binding site for Wg to Dfz2. Third, as proposed for other co-receptors¹⁵, Dally could limit the free diffusion of Wg by capturing it on the cell surface, thereby increasing its local concentration and the probability that it will interact with less abundant, high-affinity signalling receptors. Biochemical analyses between Dally, Wg and Dfz2 will be required to distinguish between these models. Interestingly, both Dfz2 and Fz encode redundant Wg receptors in the embryo¹². Thus it is possible that, in addition to having a role in the Wg/Dfz2 interaction, Dally also cooperates with Wg/Fz. Furthermore, Dally regulates the

activity of decapentaplegic (Dpp)¹⁶, a member of the TGF- β superfamily. As we have no evidence for a role for HSPGs in the early function of Dpp in the establishment of dorso-ventral embryonic polarity, the function of Dally may be tissue-specific. Tissue-specific effects of Dally could be generated either through tissue-specific expression of *dally* during development or tissue-specific modification of the heparan sulphate GAG chains linked to the Dally protein core. There is biochemical and genetic evidence to support the model that specific heparan sulphate GAGs decorate the cell surface. In vertebrates, a number of sulphotransferases are differentially expressed in various tissues¹⁷. In addition, the *Drosophila* gene *pipe*, which is involved in dorso-ventral patterning in the embryo, encodes a putative heparan sulphate 2-O sulphotransferase that is expressed in ventral follicle cells¹⁸. □

Methods

Reagents. The *sfl* alleles are *sfl*⁽³⁾⁰³⁸⁴⁴ (ref. 5) and *sfl*^{9B4} (N.P., unpublished). Both *sfl*⁽³⁾⁰³⁸⁴⁴ and *sfl*^{9B4} show similar maternal-effect phenotypes. Females with germline clones (GLCs) were generated as described⁶. All the available *dally* alleles are homozygous viable to some extent, with *dally*^{P2} and *dally* ^{Δ P188} representing the strongest alleles available¹⁰. To try to isolate a stronger loss-of-function *dally* allele, we generated a number of new *dally* alleles by P-element excisions. However, none was stronger than the original¹⁰. Other stocks are: UAS-Dfz2 (ref. 14) and UAS-Dfz2N (ref. 13), UAS-arm^{act} (ref. 19), C96 Gal4 (ref. 20). *dally* cDNA was obtained from S. Selleck¹⁰. Crumbs, Dll and Lab antibodies were obtained from E. Knust, I. Duncan and T. Kaufman, respectively.

Molecular methods. Molecular characterization of *sfl* and RNA injection were done as described for *sgf*⁶. Western blotting of Dally was performed using a polyclonal Dally antibody, a gift of H. Nakato. The dsRNA synthesis and injection were as described¹².

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Heparan sulfate proteoglycans are essential for FGF receptor signaling during *Drosophila* embryonic development

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SUMMARY

The *Drosophila* *sugarless* and *sulfateless* genes encode enzymes required for the biosynthesis of heparan sulfate glycosaminoglycans. Biochemical studies have shown that heparan sulfate glycosaminoglycans are involved in signaling by fibroblast growth factor receptors, but evidence for such a requirement in an intact organism has not been available. We now demonstrate that *sugarless* and *sulfateless* mutant embryos have phenotypes similar to those lacking the functions of two *Drosophila* fibroblast growth factor receptors, *Heartless* and *Breathless*. Moreover, both *Heartless*- and *Breathless*-dependent MAPK activation is significantly reduced in embryos which fail to synthesize heparan sulfate glycosaminoglycans. Consistent with an involvement of *Sulfateless* and *Sugarless* in fibroblast growth factor receptor signaling, a constitutively activated form of *Heartless* partially rescues

sugarless and *sulfateless* mutants, and dosage-sensitive interactions occur between *heartless* and the heparan sulfate glycosaminoglycan biosynthetic enzyme genes. We also find that overexpression of *Branchless*, the *Breathless* ligand, can partially overcome the requirement of *Sugarless* and *Sulfateless* for *Breathless* activity. These results provide the first genetic evidence that heparan sulfate glycosaminoglycans are essential for fibroblast growth factor receptor signaling in a well defined developmental context, and support a model in which heparan sulfate glycosaminoglycans facilitate fibroblast growth factor ligand and/or ligand-receptor oligomerization.

Key words: *Drosophila*, FGF, Heparan sulfate glycosaminoglycan, Mesoderm, Trachea, Cell migration

INTRODUCTION

The fibroblast growth factors (FGFs) constitute a large family of proteins that act as key intercellular signals in a wide range of developmental and pathological processes (Hanahan and Folkman, 1996; Martin, 1998; Wilkie et al., 1995; Yamaguchi and Rossant, 1995). The biological functions of FGFs include the regulation of cell proliferation, differentiation, survival, motility and tissue patterning. Both biochemical and genetic studies demonstrate that FGFs relay their signals through high affinity transmembrane protein tyrosine kinase receptors (Mason, 1994; Wilkie et al., 1995). By binding to the extracellular domains of these receptor tyrosine kinases (RTKs), FGFs induce the formation of receptor homo- or heterodimers. FGF receptor (FGFR) oligomerization results in RTK transphosphorylation followed by the activation of a Ras-dependent intracellular signal transduction pathway (Fantl et al., 1993).

In addition to its high affinity receptor, biochemical studies indicate that heparin/heparan sulfate proteoglycans (HSPGs) act as low affinity FGF co-receptors that facilitate FGF signal transduction (Mason, 1994; Ornitz et al., 1992; Rapraeger et

al., 1991; Schlessinger et al., 1995; Spivak-Kroizman et al., 1994; Yayon et al., 1991). HSPGs are ubiquitous macromolecules that are associated with the cell surface and with the extracellular matrix (Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). HSPGs consist of a protein core to which heparin/heparan sulfate glycosaminoglycan (HS GAG) is attached. The function of HSPGs in FGF signaling is mediated by HS GAG chains which are usually highly sulfated and negatively charged. While the precise mechanism by which HS GAGs participate in FGFR activation remains unclear, a variety of biochemical studies suggest that HS GAGs may stabilize or induce the formation of FGF dimers or a ternary complex composed of ligand plus high and low affinity receptors (DiGabriele et al., 1998; Faham et al., 1996; Herr et al., 1997; Ornitz et al., 1992, 1995; Spivak-Kroizman et al., 1994; Venkataraman et al., 1996; Zhu et al., 1993). Although there is strong in vitro evidence implicating HS GAGs in FGFR signaling, there is as yet no in vivo genetic support for this hypothesis.

Two FGFRs, *Heartless* (Htl) and *Breathless* (Btl), have been characterized in *Drosophila*. Genetic analyses have established

that each of these receptors has distinct expression patterns and developmental functions during embryogenesis. Htl is expressed uniformly in the early embryonic mesoderm where it is required for the normal dorsolateral migration of mesodermal cells following gastrulation (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998b; Shishido et al., 1993, 1997). Htl expression is modulated after mesoderm migration is complete, and its continued activity is essential for the specification of particular cardiac and muscle cell fates (Carmena et al., 1998; Michelson et al., 1998b). Btl is expressed in the tracheal system as well as in a subset of cells in the CNS midline (Klämbt et al., 1992; Shishido et al., 1993). Both of these cell types depend on Btl for their specific patterns of migration. For example, in *btl* mutant embryos, the primary specification of tracheal cells is normal but these cells fail to migrate properly, leading to profound defects in the formation of the tracheal tree (Klämbt et al., 1992). Btl also is required for the determination of specialized cells at the ends of primary tracheal branches that initiate the formation of higher order branching (Lee et al., 1996; Reichman-Fried and Shilo, 1995). While the ligand for Htl has not yet been identified, the Btl ligand is encoded by *branchless* (*bnl*; Sutherland et al., 1996). Mutations in *bnl* are associated with defects in tracheal morphogenesis that are virtually identical to those seen in *btl* mutants. Bnl is expressed dynamically in clusters of cells that are positioned so as to guide the outgrowth and subsequent branching of neighboring tracheal cells. Ectopic expression of Bnl can redirect tracheal cell outgrowth and branch formation, substantiating the hypothesis that spatially localized activation of the Btl receptor is necessary for normal tracheal morphogenesis (Lee et al., 1996; Sutherland et al., 1996). The characterization of FGFRs and their ligands by a combination of genetic as well as molecular approaches in *Drosophila* provides a very useful paradigm for studying the biological functions of FGFs and for identifying other genetic components involved in FGF signaling.

We have recently identified and characterized two *Drosophila* mutations, *sugarless* (*sgl*, also known as *kiwi* and *suppenkasper*; Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997) and *sulfateless* (*sfl*; Lin and Perrimon, 1999) which encode the homologs of UDP-D-glucose dehydrogenase and heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively. These enzymes are critical for the biosynthesis and modification of HS GAGs, and the corresponding mutants provide an *in vivo* model for examining the involvement of these molecules in FGFR signaling. In particular, we have used these mutants to test the hypothesis that HSPGs act as FGF co-receptors by determining whether *sfl* or *sgl* null embryos exhibit phenotypes characteristic of the high affinity FGFR mutants, *htl* and *btl*, whether *sfl* and *sgl* interact genetically with *htl*, and whether Htl- and Btl-dependent signaling pathways are activated in *sfl* and *sgl* null embryos. In this report, we demonstrate that the complete loss of both the maternal and zygotic components of either *sfl* or *sgl* expression is associated with profound defects in mesoderm and tracheal cell migration. Moreover, both the Htl- and Btl-dependent activation of MAPK that is seen normally in migrating mesodermal and tracheal cells is undetectable in *sfl* and *sgl* mutant embryos. These findings provide the first genetic evidence that HS GAGs are essential for signaling by FGFRs during embryonic development.

MATERIALS AND METHODS

Drosophila strains

The following *Drosophila* strains were employed: *htl*^{AB42}, *htl*^{YY262} (Gisselbrecht et al., 1996; Michelson et al., 1998a), *sfl*^{l(3)03844} (Lin and Perrimon, 1999), *sgl*^{l(3)08310} (Häcker et al., 1997), *btl*^{LG19} (Klämbt et al., 1992), *bnl*^{P1} (Sutherland et al., 1996), *wg*^{CX4} (Bejsovec and Wieschaus, 1993; van den Heuvel et al., 1993), *69B-Gal4* (Brand and Perrimon 1993), *twi-Gal4* (Greig and Akam, 1993), UAS-Bnl (Sutherland et al., 1996), UAS-Htl^{Act} (Michelson et al., 1998a) and *trh-lacZ* (Wilk et al., 1996). *htl*^{AB42}, *sfl*^{l(3)03844}, *sgl*^{l(3)08310}, *btl*^{LG19}, *bnl*^{P1} and *wg*^{CX4} are all null alleles by genetic and/or molecular criteria. *Oregon R* was used as a wild-type reference strain.

Generation of females with *sfl* and *sgl* germline clones

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype *sfl* (or *sgl*) *FRT*^{2A}/*TM3*, *Sb* were mated with males of the genotype *y w FLP*^{22/+}, *FRT*^{2A} *P[ovoD1]*/*TM3*, *Sb*. The resulting progeny were heat shocked at 37°C for 2 hours during the larval stages of development, and *y w FLP*^{22/+}; *sfl* (or *sgl*) *FRT*^{2A}/*FRT*^{2A} *P[ovoD1]* females carrying *sfl* (or *sgl*) homozygous germline clones were selected. Such females were mated to *sfl* (or *sgl*)/*TM3-ftz lacZ* males, and maternal/zygotic null embryos were identified by the absence of *lacZ* expression.

Antibody staining

Fixation of embryos, antibody staining and embryo sectioning were performed as described by Gisselbrecht et al. (1996); Michelson et al. (1998a); Patel (1994). Anti-tracheal lumen antibody mAb2A12 was obtained from the Developmental Studies Hybridoma Bank, anti- β -galactosidase antibody from Cappel and Promega, and anti-diphospho-MAPK antibody from Sigma.

Ectopic expression experiments

Targeted ectopic expression was accomplished using the Gal4/UAS system (Brand and Perrimon 1993). Chromosomes bearing Gal4 and UAS insertions were combined with appropriate mutations using standard genetic crosses. Mesodermal and ectodermal expression were achieved with *twi*-Gal4 and *69B*-Gal4 lines, respectively, in both wild-type and mutant genetic backgrounds.

Quantitation of mesodermal phenotypic severity

The hypomorphic *htl*^{YY262} mutation was recombined with null alleles of *sfl* and *sgl*. Embryos from stocks containing these recombinant chromosomes maintained over a *lacZ*-marked balancer were collected and double stained with antibodies against both Eve and β -galactosidase. Embryos homozygous for the zygotic loss of both genes were identified as lacking *lacZ* expression. Identical experiments were undertaken with each of the single zygotic mutants, and Eve expression in dorsal mesodermal cells was quantitated for each genotype, as previously described (Michelson et al., 1998a,b). The statistical significance of the difference in Eve expression for each pairwise comparison was calculated using both one-tailed *z*- and *t*-tests.

RESULTS

The genetics of HSPG biosynthesis in *Drosophila*

In a screen to characterize the maternal effects of zygotic lethal mutations (Perrimon et al., 1996), two mutants, *sfl* and *sgl*, were isolated on the basis of their abnormal embryonic segmentation phenotypes. Embryos lacking maternal germline-derived *sfl* or *sgl* activity, as well as the paternally

derived zygotic activity of these genes (referred to hereafter as *sfl* or *sgl* null embryos), exhibit a cuticle phenotype similar to that of the *wingless* (*wg*) segment polarity mutant (Binari et al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999). *sgl* encodes a homolog of bovine UDP-glucose dehydrogenase (Hempel et al., 1994) which catalyzes the conversion of UDP-D-glucose to UDP-D-glucuronic acid, an essential substrate for GAG biosynthesis. Consistent with a critical role for Sgl in the synthesis of proteoglycans in *Drosophila*, prior biochemical experiments have established that Syndecan and Dally/Glypican lack GAG chains in *sgl* null embryos (Haerry et al., 1997) or homozygous *sgl* zygotic mutant third instar larvae (Tsuda et al., 1999), respectively. Moreover, injection of either UDP-glucuronic acid or heparan sulfate into *sgl* null embryos rescues the *wg*-like segment polarity defect, and injection of heparinases I and III (but not chondroitinase ABC) into wild-type embryos phenocopies loss of *sgl* function (Binari et al., 1997). These findings provide strong evidence that Sgl is essential for the biosynthesis of HS GAG chains which, in turn, are required for Wg signaling.

Drosophila Sfl (Lin and Perrimon, 1999) has 51% and 53% amino acid identity to rat and mouse heparin/heparan sulfate N-deacetylase/N-sulfotransferase, respectively (Eriksson et al., 1994; Hashimoto et al., 1992; Orellana et al., 1994). This enzyme catalyzes the N-deacetylation and N-sulfation of polymerized heparan (as opposed to chondroitin or dermatan) GAGs, the key step that initiates further GAG modifications (for review see Kjellén and Lindahl, 1991). Since the sulfation and epimerization of HS GAGs provide structural identity as well as the negative charge that is critical for their interaction with proteins, loss of Sfl activity will result in the generation of unmodified HS GAGs, thereby impairing normal HSPG functions. Indeed, similar to the case in *sgl* mutants (Tsuda et al., 1999), the HS GAG-modified Dally protein is markedly reduced in *sfl* zygotic mutant larvae, indicating that, in the absence of Sfl activity, HS GAG biosynthesis is abnormal (Lin and Perrimon, 1999). Together with the strong *wg*-like segment polarity phenotype associated with loss of *sfl* function, these results provide compelling evidence that Sfl is essential for the production of proteoglycans involved in Wg signaling (Lin and Perrimon, 1999). Moreover, the specificity of Sfl for heparan-containing GAGs distinguishes the type of GAG chain that participates in this signaling pathway. In summary, phenotypic analyses of *sfl* and *sgl* mutants can be used to study the roles of HSPGs in normal developmental processes.

sfl and *sgl* mutants phenocopy the mesoderm migration defect associated with loss of *htl* function

After invaginating through the ventral furrow at gastrulation, Twist- (*Tw*) expressing mesodermal cells migrate along the ectoderm in a dorsolateral direction. By late stage 9, the mesoderm is composed of an inner monolayer of cells that extends from the ventral midline to the dorsal edge of the ectoderm (Bate, 1993; Leptin, 1995; Fig. 1A,E). In *htl* mutant embryos, gastrulation is normal but mesoderm migration fails to occur properly, resulting in an irregular dorsal margin of *Tw*-positive cells and a relative accumulation of these cells in ventral and lateral positions (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997; Michelson et al., 1998b; Fig. 1B,F). A similar phenotype occurs in *sfl* and *sgl* null embryos (Fig. 1C,D,G,H). Of note, mesoderm migration is normal in *wg* mutant embryos (data not shown), suggesting that this effect of *sfl* and *sgl* is not due to an influence on Wg signaling, as is the case for the segmentation effects of these genes (Binari et al.

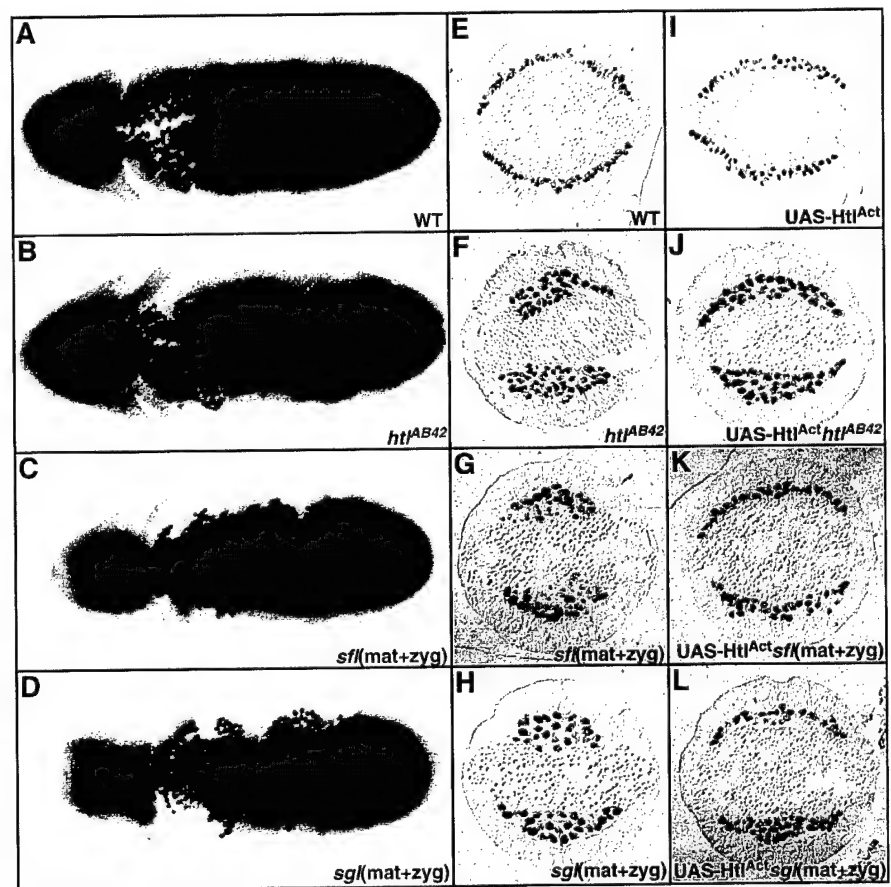


Fig. 1. *sfl* and *sgl* are required for the dorsolateral migration of the embryonic mesoderm, a requirement that is partially bypassed by constitutive *Htl* signaling. (A-D) Ventral views of late stage 9 embryos of the indicated genotypes immunostained for *Tw* expression. *mat+zyg*: an embryo from a germline clone female that has also inherited a mutant paternal chromosome. These embryos therefore lack both the maternal and zygotic functions of *sfl* or *sgl*. (E-H) Transverse sections of *Tw*-stained early stage 10 embryos of the indicated genotypes. Whereas *Tw*-positive mesodermal cells have reached the dorsal ectoderm in wild type, mesoderm migration fails to occur properly in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. (I-L) *twi*-Gal4-mediated ectopic expression of a constitutively activated form of *Htl* partially rescues the mesoderm migration defects of null *htl* and maternal/zygotic null *sfl* and *sgl* embryos, but has no effect on migration in an otherwise wild-type genetic background (I).

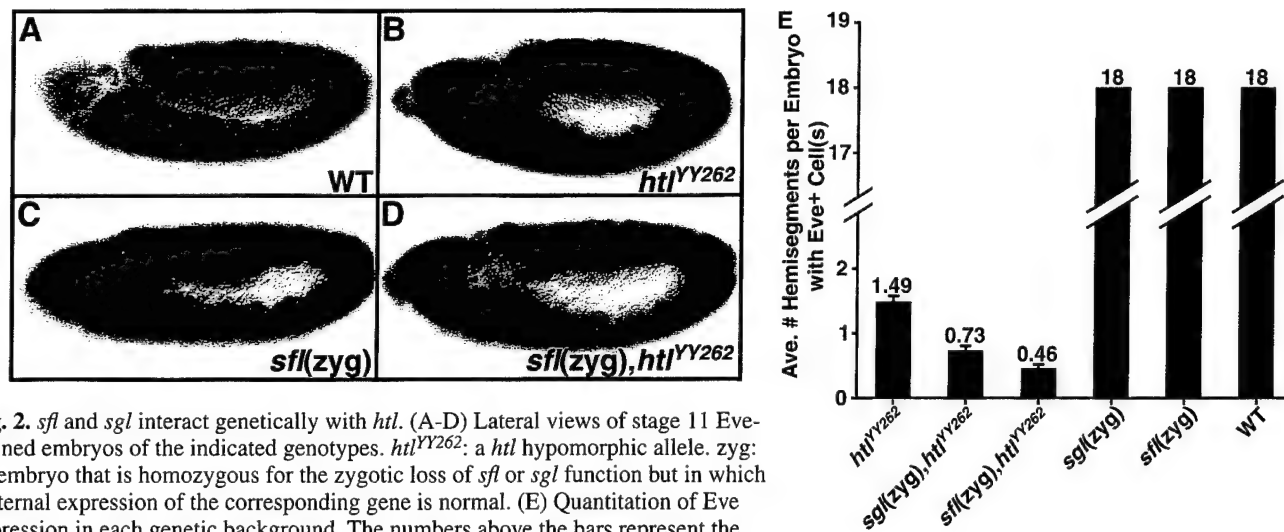


Fig. 2. *sfl* and *sgl* interact genetically with *htl*. (A–D) Lateral views of stage 11 *Eve*-stained embryos of the indicated genotypes. *htl^{YY262}*: a *htl* hypomorphic allele. *zyg*: an embryo that is homozygous for the zygotic loss of *sfl* or *sgl* function but in which maternal expression of the corresponding gene is normal. (E) Quantitation of *Eve* expression in each genetic background. The numbers above the bars represent the average number of hemisegments per embryo of the indicated genotypes in which *Eve*-positive cells develop. Each error bar indicates the standard error of the mean for that genotype. *Eve* expression was scored in the second and third thoracic and first seven abdominal segments on both sides of each embryo. At least 80 embryos of each genotype were scored. The *Eve* pattern is unaltered by zygotic loss of either *sfl* or *sgl* due to the normal maternal expression of these genes. In a *htl* hypomorph, only a partial reduction in *Eve* expression occurs, a phenotype that is significantly ($P < 10^{-5}$) enhanced by the zygotic loss of either *sfl* or *sgl*.

al., 1997; Häcker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999).

The finding that mesoderm migration depends on *sfl* and *sgl*, as well as on *htl*, raises the possibility that HS GAG biosynthesis is required for signaling by the Htl FGFR. If, as has been suggested, HS GAGs participate in the activation of FGFRs (Ornitz et al., 1992; Rapraeger et al., 1991; Schlessinger et al., 1995; Yayon et al., 1991), then a constitutively active form of Htl should bypass the requirement of *sfl* and *sgl* for mesoderm migration. We tested this hypothesis by targeting the expression of activated Htl to the mesoderm of *sfl* or *sgl* null embryos using the Gal4/UAS system (Brand and Perrimon, 1993). We previously reported the construction of such an activated receptor in which the extracellular domain of wild-type Htl is replaced by the dimerization domain of the bacteriophage λ cI repressor (Michelson et al., 1998a). As with other RTKs, this manipulation generates constitutive, ligand-independent receptor activity (Lee et al., 1996; Michelson et al., 1998a; Queenan et al., 1997). In otherwise wild-type embryos, *twi*-Gal4-mediated ectopic expression of activated Htl has no significant effect on the migration of mesodermal cells (Michelson et al., 1998a; Fig. 1I). However, activated Htl is able to weakly restore mesoderm migration in a null *htl* mutant (Michelson et al., 1998a; Fig. 1J). Similarly, activated Htl partially rescues the migration defect of *sfl* and *sgl* null embryos (Fig. 1K,L). Only partial rescue is seen in all mutant backgrounds due to the relatively weak constitutive activation of Htl that is achieved by spontaneous dimerization (Michelson et al., 1998a). In addition, constitutive Htl does not reproduce the graded activity of this receptor that occurs during normal mesoderm migration (Gabay et al., 1997b; Michelson et al., 1998a). The timing of constitutive Htl expression induced by *twi*-Gal4 is unlikely to be a contributing factor since the equivalent expression of wild-type Htl completely rescues a null *htl* allele (Michelson et al., 1998b). In summary, these

genetic epistasis experiments suggest that Htl acts downstream of Sfl and Sgl in migrating mesodermal cells.

If HS GAGs participate in the activation of Htl, then *sfl* and *sgl* should exhibit dosage-sensitive genetic interactions with *htl*. We investigated this possibility using a quantitative assay that is based on the expression of *even skipped (eve)* in dorsal mesodermal progenitor cells (Buff et al., 1998; Carmena et al., 1998; Frasch et al., 1987; Michelson et al., 1998a,b). The development of these *Eve*-expressing cardiac and somatic muscle cells depends on Htl for both mesoderm migration and cell fate specification. In wild-type embryos, *Eve* is found in segmentally repeated groups of cells that are confined to the dorsal mesoderm (Fig. 2A). All such cells are missing in a null *htl* mutant (Gisselbrecht et al., 1996). However, in a *htl* hypomorph, mesoderm migration and subsequent cell fate specification are only partially disrupted, permitting some dorsal *Eve* expression to occur (Fig. 2B; Michelson et al., 1998b). In embryos in which only the zygotic activity of *sfl* or *sgl* is absent, *Eve* expression is entirely normal, reflecting the strong maternal contribution of these genes (Fig. 2C and data not shown). However, when complete zygotic loss of *sfl* or *sgl* is combined with homozygosity for the *htl* hypomorphic allele, the severity of the *Eve* phenotype is enhanced (Fig. 2D). Quantitation of the number of *Eve*-positive hemisegments in *htl*, *sfl*, *sgl*, *htl sfl* and *htl sgl* embryos demonstrated that the interactions between *htl* and both *sfl* and *sgl* are highly significant (Fig. 2E; $P < 10^{-5}$). These results, combined with the above findings that *htl* is epistatic to *sfl* and *sgl*, are consistent with the hypothesis that HS GAGs are essential for Htl activation during mesoderm migration in the *Drosophila* embryo.

***sfl* and *sgl* are required for Btl-dependent tracheal cell migration**

Given the genetic evidence that Sfl and Sgl are required for Htl FGFR signaling in the mesoderm, we next determined whether

HS GAG biosynthesis is also involved in signaling by Bnl and Btl during trachea development. Since Wg function is compromised in *sgl* and *sfl* null mutant embryos, and *wg* loss-of-function itself leads to abnormal tracheal morphogenesis secondary to an accompanying segmentation defect (see below), it is difficult to solely correlate the tracheal cell migration phenotype of *sgl* and *sfl* null mutants with Bnl/Btl signaling. We therefore analyzed tracheal cell migration in embryos deficient only in the zygotic functions of *sfl* and *sgl*. Segmentation and the expression of Engrailed are normal in such embryos, indicating that Wg signaling is unaffected (Perrimon et al., 1996; Häcker et al., 1997; Lin and Perrimon, 1999).

The tracheal system of the *Drosophila* embryo forms by a sequential series of branching steps that can be visualized by following the expression of an enhancer trap in the *trachealess* (*trh*) gene (Isaac and Andrew, 1996; Wilk et al., 1996; Fig. 3A). In stage 13 embryos homozygous for loss of the zygotic functions of either *sfl* or *sgl*, the early steps in tracheal branching are significantly perturbed (Fig. 3B,C). By late stage 15, wild-type embryos have developed an extensive tracheal network in which the dorsal and lateral trunk branches have fused and additional primary and secondary branches have formed (Samakovlis et al., 1996; Fig. 3D). In either *sfl* or *sgl* zygotic mutants, tracheal branch formation is incomplete, as revealed by the presence of large gaps in the dorsal and lateral trunks, as well as stalled ganglionic branches (Fig. 3E,F). The penetrance of this phenotype is incomplete and the expressivity is variable in both *sfl* and *sgl* zygotic mutants; 16% of *sfl* ($n=245$) and 7% of *sgl* ($n=198$) zygotic mutant embryos exhibit some degree of abnormal tracheal morphogenesis, ranging from one to all segments having breaks in the dorsal trunk. In contrast, in *btl* and *bnl* null mutant embryos, tracheal cell migration is more severely affected and virtually no branches form from the initial tracheal invaginations (Klämbt et al., 1992; Sutherland et al., 1996; Fig. 3G). The partial disruption of the tracheal tree seen with zygotic loss of *sfl* and *sgl* is reminiscent of the defects observed in hypomorphic mutants of *btl*, *bnl* and *heartbroken* (*hbr*)/downstream of FGF receptor (*dof*)/stumps, a gene that encodes a specific effector of FGF receptor signaling (Klämbt et al., 1992; Michelson et al., 1998a; Sutherland et al., 1996; Vincent et al., 1998; Imam et al., 1999). The relatively weak zygotic *sfl* and *sgl* tracheal phenotypes are most likely due to partial rescue by the maternal expression of these genes, as previously noted for their mesodermal activities. The more severe tracheal defects that occur in *sfl* and *sgl* null embryos are consistent with this suggestion (see below). In summary, the present findings implicate Sfl- and Sgl-dependent HS GAG biosynthesis in signaling by the Btl FGFR.

Htl- and Btl-dependent MAPK activation depends on *sfl* and *sgl*

Htl and Btl are RTKs that transduce their intracellular signals by the conserved Ras/MAPK cascade (Cobb and Goldsmith, 1995; Seger and Krebs, 1995). As a result, RTK activity can be

visualized in developing tissues with an antibody specific for the diphosphorylated, activated form of MAPK (Gabay et al., 1997a,b). Using this approach, diphospho-MAPK is observed at the leading edge of the migrating mesoderm in wild-type embryos (Gabay et al., 1997b; Michelson et al., 1998a; Vincent et al., 1998; Fig. 4A). In either *htl* or *hbr/dof/stumps* mutants, this expression of diphospho-MAPK is undetectable (Michelson et al., 1998a; Vincent et al., 1998; Fig. 4B). Similarly, the Htl-dependent mesodermal localization of diphospho-MAPK is below the level of detection in both *sfl* and *sgl* null embryos (Fig. 4C,D). In contrast, epidermal growth factor receptor (EGFR)-dependent MAPK activation in the ventral epidermis and amnioserosa does not require either *sfl* or *sgl*, consistent with the specific involvement of these genes in FGFR signaling.

During stage 11, MAPK is activated by Btl in the tracheal pits (Gabay et al., 1997b; Fig. 4E). As expected, this expression of diphospho-MAPK is markedly reduced in *btl* and *bnl* mutant embryos (Fig. 4F,G). The FGFR-specific signal transducer Hbr/Dof/Stumps also is required for MAPK activation in the tracheal pits (Michelson et al., 1998a; Vincent et al., 1998). In contrast, in *wg* mutants MAPK activation by Btl is unaffected, although the normal spacing between the tracheal pits is reduced due to the associated segmentation defect (Fig. 4H). As with *btl*, *bnl* and *hbr/dof/stumps*, Btl-dependent MAPK activation is significantly decreased in *sfl* or *sgl* null embryos (Fig. 4I,J), whereas the earlier EGFR-dependent expression of

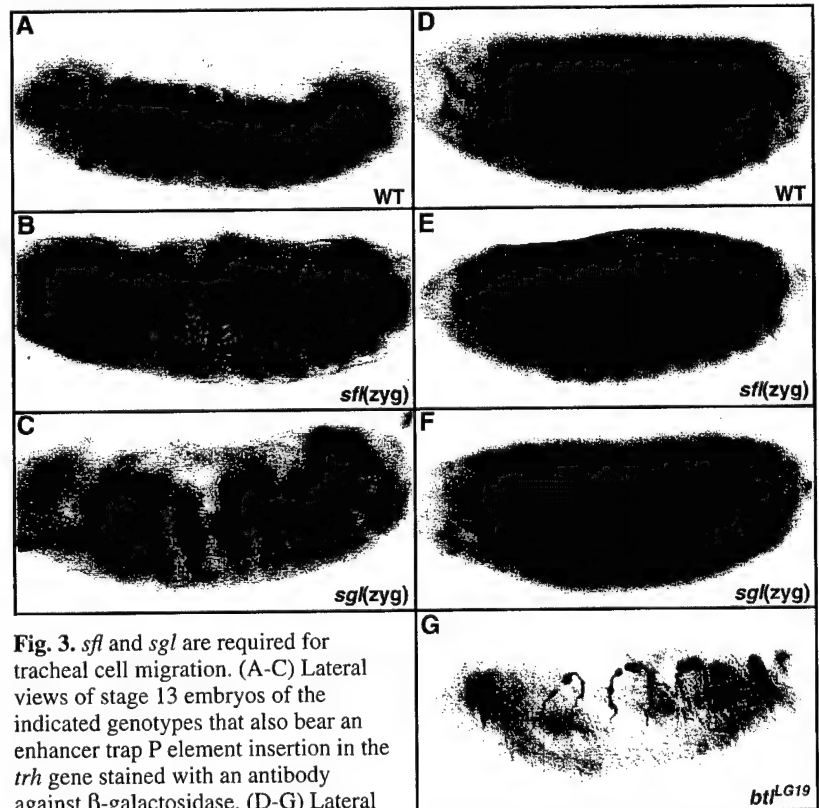


Fig. 3. *sfl* and *sgl* are required for tracheal cell migration. (A-C) Lateral views of stage 13 embryos of the indicated genotypes that also bear an enhancer trap P element insertion in the *trh* gene stained with an antibody against β -galactosidase. (D-G) Lateral views of late stage 15 embryos stained for expression of an antigen that localizes to the tracheal lumen. Tracheal cell migration is abnormal in embryos deficient in the zygotic functions of *sfl* and *sgl*. This phenotype is incompletely penetrant and its expressivity is variable (see text for details). The embryos shown in panels B,C,E and F represent phenotypes of intermediate severity.

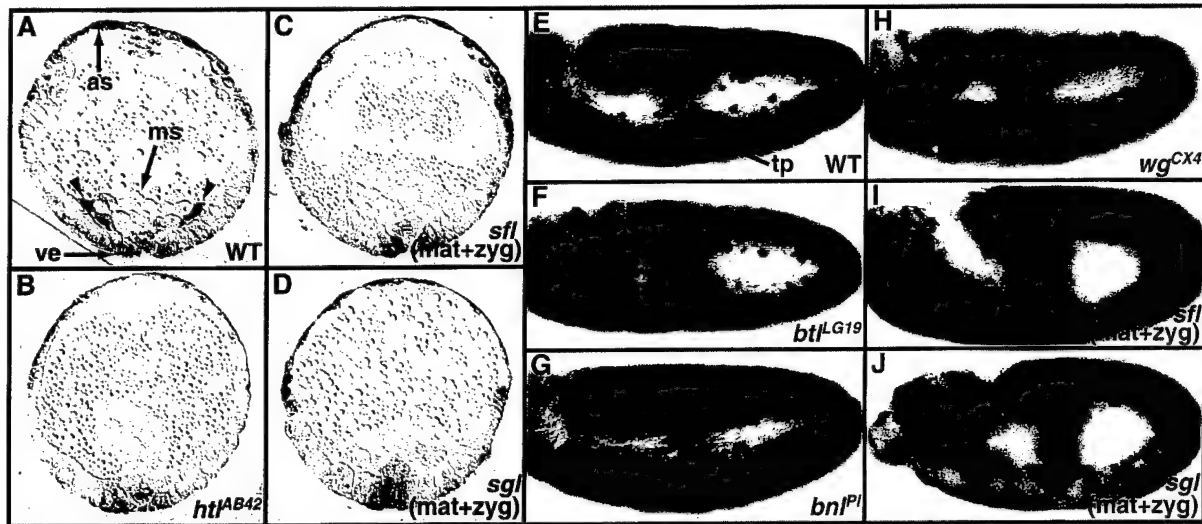


Fig. 4. *sfl* and *sgl* are essential for Htl- and Btl-dependent MAPK activation. (A-D) Transverse sections of stage 8 embryos immunostained with an antibody specific for the activated or diphosphorylated form of MAPK. In wild type, diphospho-MAPK expression is localized to the leading edge (arrowheads) of the migrating mesoderm (ms). This expression is below the level of detection in null *htl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. However, EGFR-dependent diphospho-MAPK expression in the amnioserosa (as) and ventral epidermis (ve) is not affected in any of these mutants. (E-J) Lateral views of stage 11 embryos stained with the diphospho-MAPK-specific antibody. The strong expression that is observed in wild-type tracheal pits (tp) is markedly reduced in the corresponding positions in null *btl* and *btl* as well as in maternal/zygotic null *sfl* and *sgl* embryos. In contrast, diphospho-MAPK is normally expressed in the tracheal pits of *wg* mutant embryos, although these structures are spaced more closely secondary to the *wg* segment polarity phenotype.

diphospho-MAPK in the tracheal placodes is unaffected in each of these mutants (Michelson et al., 1998a; data not shown). These in situ patterns of MAPK activation provide direct evidence that Sfl and Sgl are required for signaling by the two *Drosophila* FGFRs, Htl and Btl, independent of the requirement for HS GAGs in Wg function.

Overexpression of Bnl partially overcomes the requirement of *sfl* and *sgl* for tracheal cell migration

It has been suggested that monomeric FGF molecules are capable of self-assembling into dimers and higher order oligomers but, at physiological concentrations, require a HS GAG to stabilize this interaction (Sasisekharan et al., 1997; Venkataraman et al., 1996). If this is the case, then elevated levels of the growth factor may at least partially overcome the need for the HS GAG to generate a biological response. We tested this possibility by assessing the effects on tracheal cell migration of overexpressing Bnl, the Btl ligand, in *sfl* and *sgl* null embryos.

In an otherwise wild-type genetic background, high level ectopic ectodermal expression of Bnl hyperactivates Btl, leading to an inhibition of primary tracheal branching, as well as the overproduction of secondary and terminal branches (Sutherland et al., 1996; Michelson et al., 1998a; Fig. 5A,B). These effects of ectopic Bnl are weakly suppressed in the absence of zygotic *sfl* and *sgl* functions (Fig. 5C,D). In embryos lacking both the zygotic and maternal components of *sfl* and *sgl* expression, virtually no tracheal branches are observed (Fig. 5E,F). This correlates very well with the marked reduction in diphospho-MAPK expression that is seen at earlier stages in *sfl* and *sgl* null embryos (Fig. 4I,J). However, in *sfl* and *sgl* null embryos in which Bnl is ectopically expressed, there is a partial recovery of tracheal branching (Fig. 5G,H). Although the tracheal phenotype of *sfl* and *sgl* null embryos reflects the combined loss of Wg and Btl signaling, it is

noteworthy that tracheal morphogenesis is not completely inhibited in *wg* as it is in null *sfl* and *sgl* mutants (compare Fig. 5E,F and I). Moreover, ectopic Bnl in the absence of *wg* function leads to a very marked increase in fine tracheal branching, very similar to the effect of ectopic Bnl in wild-type embryos (compare Fig. 5B and J). Thus, the severe tracheal phenotype associated with complete loss of *sfl* or *sgl* function primarily is attributable to an involvement of HS GAGs in Btl rather than in Wg signaling. The ability of Bnl overexpression to partially bypass the requirement for Sfl and Sgl is therefore consistent with a role for HS GAGs in stabilizing or facilitating FGF self-association (Zhu et al., 1993; Ornitz et al., 1995; Faham et al., 1996; Venkataraman et al., 1996; Sasisekharan et al., 1997; DiGabriele et al., 1998). Such a HS GAG-FGF dimer complex would, in turn, facilitate the dimerization of high affinity FGFRs, a prerequisite for receptor activation and the transmission of intracellular signals.

DISCUSSION

We have shown that two enzymes involved in the biosynthesis of HS GAGs are essential for signaling by both of the known *Drosophila* FGFRs, Htl and Btl. Loss of either *sfl* or *sgl* function leads to defects in the migration of mesodermal and tracheal cells during embryogenesis. In addition, Htl- and Btl-dependent activation of MAPK is markedly reduced in *sfl* and *sgl* null embryos. Taken together, these findings provide the first genetic evidence that HSPGs play a central role in FGFR signaling in a well-defined developmental context. A similar genetic approach has been used to establish that HSPGs are critical components of Wg signaling in *Drosophila* (Binari et al., 1997; Häcker et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999).

Several mechanisms have been proposed for how HSPGs participate in FGFR signaling. In one model, the binding of

FGF to abundant but low affinity HS GAGs on the cell surface limits the free diffusion of the ligand, thereby increasing its local concentration and the probability that it will interact with less abundant, high affinity signaling receptors (Schlessinger et al., 1995). Other studies have identified distinct HS GAG binding sites on both FGF (Blaber et al., 1996; Eriksson et al., 1991; Zhang et al., 1991) and high affinity FGFRs (Kan et al., 1993; Pantoliano et al., 1994), suggesting that the latter two components form a ternary complex with a proteoglycan. Direct interaction between the proteoglycan and FGFR, in addition to growth factor binding, may therefore be required for maximal receptor activation. HS GAGs also may promote the formation of FGF dimers or higher order oligomers, thereby facilitating FGFR dimerization and activation (Zhu et al., 1993; Spivak-Kroizman et al., 1994; Ornitz et al., 1995; Faham et al., 1996; Digabriele et al., 1998).

A variation of the last model proposes that FGF monomers are capable of self-associating, a process that is stabilized by HS GAGs (Venkataraman et al., 1996; Herr et al., 1997; Sasisekharan et al., 1997). This hypothesis predicts that elevated levels of FGF should compensate at least in part for a loss of dimer stabilization mediated by HS GAGs. The ability of Bnl overexpression to induce some tracheal branching in the complete absence of Sfl and Sgl activities is consistent with this last possibility, although it does not rule out an additional involvement of HS GAGs or the core protein of a proteoglycan in some other aspect of Btl activation. In the case of human FGF-2, self-association in the absence of HS GAGs has been observed by mass spectrometry and biochemical assays at physiological concentrations of ligand. In addition, elevated FGF-2 levels exert biological effects on tissue culture cells that fail to synthesize HS GAGs (Davis et al., 1999). Our Bnl overexpression experiments are in agreement with the latter data for FGF-2. Together, these findings support the proposal that HS GAGs function to stabilize the FGF dimers or higher order oligomers that are formed by a self-association mechanism (Venkataraman et al., 1996; Sasisekharan et al., 1997). Interestingly, ectopic expression of ligand also is able to overcome the requirement of HS GAGs in Wg signaling, although in this case the HS GAGs may be more important for increasing the local concentration of growth factor at the cell surface than for facilitating ligand dimerization (Häcker et al., 1997; Lin and Perrimon, 1999).

Under normal conditions, the concentrations of FGFs may be limiting, necessitating the presence of HS GAGs to augment or stabilize ligand dimerization and subsequent FGFR activation. This is of particular significance for Btl signaling since *bnl*, which encodes its ligand, is known to be haploinsufficient (Sutherland et al., 1996), and

overexpression of Bnl partially bypasses the requirement for *sfl* and *sgl* in the promotion of tracheal branching. Thus, HS GAGs may ensure that a requisite FGFR activation threshold is surpassed when the amount of available ligand is normally low. Such a mechanism could additionally expand the sensitivity or spectrum of responses that can be achieved by small localized

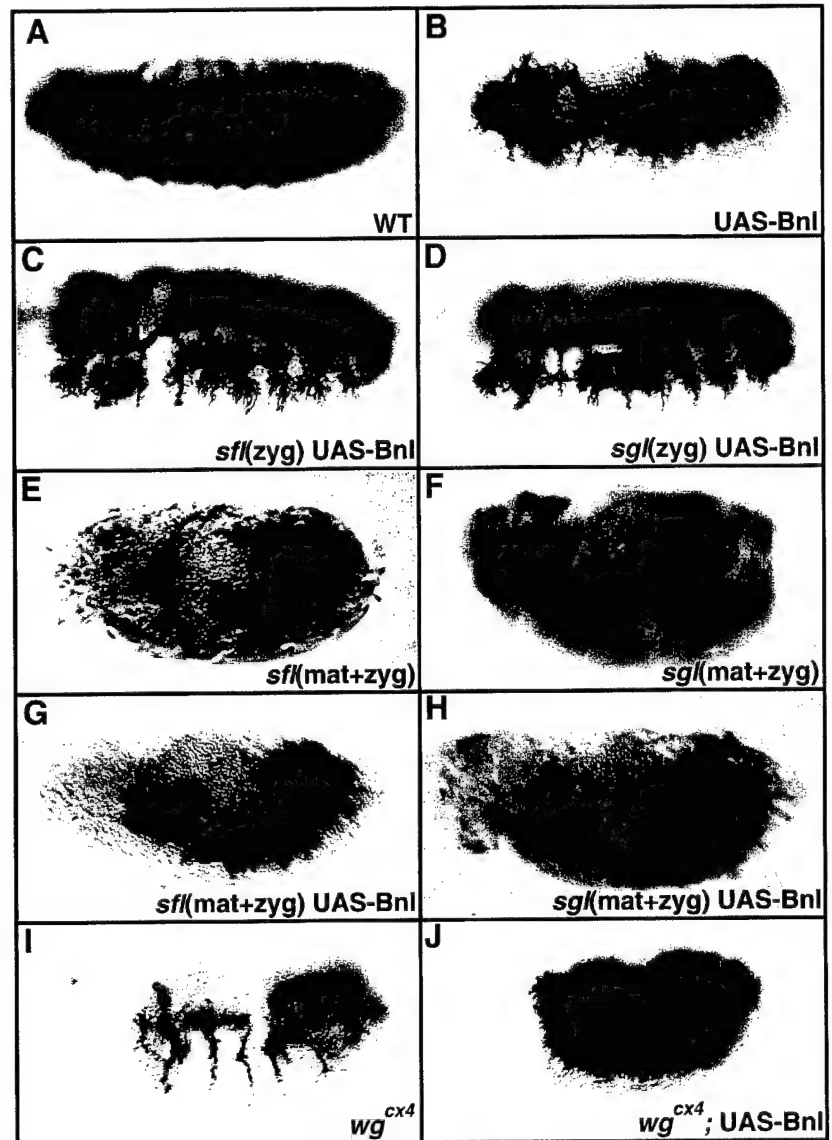


Fig. 5. Overexpression of Bnl partially rescues tracheal cell migration in *sfl* and *sgl* maternal/zygotic null mutant embryos. Lateral views of late stage 15 embryos immunostained with an antibody against a tracheal luminal antigen. (A) Wild-type tracheal pattern. In B,C,D,G,H and J Bnl was ectopically expressed throughout the ectoderm of embryos of the indicated genotypes using the *69B*-Gal4 driver. (B) Tracheal branching is markedly perturbed by ectopic Bnl in an otherwise wild-type embryo. Primary branching is suppressed and an overabundance of secondary and terminal branches is induced. (C,D) In embryos lacking only the zygotic component of *sfl* or *sgl*, the effects of ectopic Bnl are weakly blocked. (E,F) Virtually no tracheal branching occurs in maternal/zygotic null *sfl* and *sgl* embryos. (G,H) Some tracheal branching is recovered in maternal/zygotic null *sfl* and *sgl* embryos in which Bnl is ectopically expressed at high levels. (I) *wg* mutants develop an extensive tracheal network which has an abnormal pattern due to the associated segmentation defect. (J) Ectopic Bnl in a *wg* mutant background leads to an overproduction of fine tracheal branches, much as occurs in wild type (compare with B).

differences in growth factor concentrations. This is particularly relevant to Bnl which is expressed in a highly dynamic pattern during normal tracheal morphogenesis, a pattern that, when perturbed, leads to severe defects in tracheal outgrowth (Sutherland et al., 1996). Local differences in Btl activity also dictate the sites at which secondary branches normally form, a process to which HSPG regulation might contribute by generating a zone of cells that are highly responsive to Bnl in the vicinity of the ligand signaling center (Hacohen et al., 1998). Similarly, HSPG-mediated ligand dimerization could play a role in the graded activation of Htl that occurs during embryonic mesoderm migration (Gabay et al., 1997b). Modulation of RTK signaling strength also has been implicated in the generation of mesodermal progenitor identities (Buff et al., 1998).

There are two well characterized HSPGs in *Drosophila*, Dally, a Glypican-like cell surface molecule that has been implicated in both Decapentaplegic and Wg signaling (Nakato et al., 1995; Lin and Perrimon, 1999; Tsuda et al., 1999), and a transmembrane proteoglycan related to the vertebrate Syndecan family (Spring et al., 1994). It has been suggested that syndecans participate in signaling by vertebrate FGFRs (Bernfield et al., 1992; Dealy et al., 1997; Steinfeld et al., 1996), although other HSPGs may also be involved in this process (Aviezer et al., 1994, 1997; Sherman et al., 1998; Steinfeld et al., 1996). It is also possible that different HSPGs could be specific for particular FGF ligand-receptor combinations in individual tissues or at distinct developmental stages. Genetic analysis in *Drosophila* should provide a useful approach for addressing these important questions.

The present findings provide new insight into the mechanisms that regulate FGFR signaling in vivo. A more complete understanding of the function of HSPGs in Htl and Btl activation must await definitive identification of the specific proteoglycans involved, as well as structural and biochemical studies of the complexes formed between the high and low affinity receptors together with the corresponding ligands.

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The *Drosophila* *sugarless* gene modulates Wingless signaling and encodes an enzyme involved in polysaccharide biosynthesis

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SUMMARY

We have identified and characterized a *Drosophila* gene, which we have named *sugarless*, that encodes a homologue of vertebrate UDP-glucose dehydrogenase. This enzyme is essential for the biosynthesis of various proteoglycans, and we find that in the absence of both maternal and zygotic activities of this gene, mutant embryos develop with segment polarity phenotypes reminiscent to loss of either Wingless or Hedgehog signaling. To analyze the function of *Sugarless* in cell-cell interaction processes, we have focused our analysis on its requirement for Wingless signaling in different tissues. We report that *sugarless* mutations impair signaling by Wingless, suggesting that proteoglycans con-

tribute to the reception of Wingless. We demonstrate that overexpression of Wingless can bypass the requirement for *sugarless*, suggesting that proteoglycans modulate signaling by Wingless, possibly by limiting its diffusion and thereby facilitating the binding of Wingless to its receptor. We discuss the possibility that tissue-specific regulation of proteoglycans may be involved in regulating both Wingless short- or long-range effects.

Key words: *Drosophila*, Wingless, proteoglycan, *sugarless*, cell signalling

INTRODUCTION

Drosophila Wingless (Wg) belongs to a family of highly conserved proteins, the Wnt family, which have been implicated in both embryonic development and the regulation of cell proliferation in diverse species (reviewed by Nusse and Varmus, 1992; McMahon, 1992). Wnt proteins are putative secreted glycoproteins that serve as signaling molecules in intercellular communication processes to control cell differentiation and proliferation. During *Drosophila* embryonic development, Wg plays a critical role in patterning the entire segmental unit to specify ventral naked cuticle and cell type diversity in the epidermis. In addition, Wg is required for other developmental processes including segmental patterning of the midgut epithelium, development of Malpighian tubules, formation of the stomatogastric nervous system, specification of a subset of neuroblasts and imaginal disc patterning (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994).

Biochemically, Wnt family proteins possess a putative signal peptide, consensus sites for the attachment of N-linked oligosaccharides and 22 highly conserved cysteine residues, whose spacing is conserved. All the Wnt proteins examined to date enter the secretory pathway and are modified with N-linked oligosaccharides (Burrus and McMahon, 1995). Wnt proteins are poorly secreted into the culture medium and levels of the proteins are increased by addition of the polyanions heparin or suramin (Burrus and McMahon, 1995), suggesting that the majority of secreted Wnt proteins are associated with

the cell surface or the extracellular matrix (ECM). It has been demonstrated that both Wg and mouse Wnt-1 are tightly associated with the cell surface (Papkoff and Schryver, 1990; van den Heuvel et al., 1993; Reichsman et al., 1996) and the ECM (Bradley and Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993; Reichsman et al., 1996). The nature of this association is likely due to the interaction of the Wg/Wnt-1 proteins with heparan sulfate proteoglycans (HSPGs), since both of these proteins can be released by addition of exogenous heparin (Bradley and Brown, 1990; Reichsman et al., 1996). Further, heparin can directly bind to purified Wnt-1 (Bradley and Brown, 1990) and Wg protein (Reichsman et al., 1996). The association of Wg protein with the cell surface and the ECM has been shown to be important for its signaling activity, since Wg signaling can be inhibited by removal of the heparan sulfate with heparinase or by treatment of cells with sodium perchlorate, a competitive inhibitor that blocks the sulfation of proteoglycans (PGs) (Reichsman et al., 1996). Thus, HSPGs may play an important role in both the localization of Wg protein and its signaling activity during development.

While biochemical studies of Wnt signaling have been hampered by the difficulty of isolating active Wnt proteins, genetic studies in *Drosophila* have successfully identified some of the molecules involved in Wg signaling. Phenotypic and genetic interaction studies have identified four genes, *zeste white 3* (*zw3*, also known as *shaggy*), *dishevelled* (*dsh*), *armadillo* (*arm*) and *porcupine* (*porc*), which act in Wg signaling (reviewed by Perrimon, 1996). The current model is that *Porc* is required for the secretion of Wg protein, whereas

in the receiving cells, Wg initiates a signaling cascade through Dsh, which inactivates Zw3 kinase. Inactivation of Zw3 results in the accumulation of cytoplasmic Arm protein, which in turn regulates many downstream effectors of Wg, including the transcription factor *engrailed* (*en*) in the embryonic epidermis and genes of the *achaete scute complex* at the wing margin (reviewed by Siegfried and Perrimon, 1994; Klingensmith and Nusse, 1994). Recently, compelling evidence has been obtained in tissue culture cells that *Drosophila Frizzled2* (*Dfz2*) functions as a Wg receptor in cultured cells (Bhanot et al., 1996). *Dfz2* is a member of the Frizzled family of proteins, which possess a putative signal sequence, an extracellular domain composed of a conserved region of 120 amino acid residues with an invariant pattern of ten cysteines, seven putative transmembrane domains and a short cytoplasmic domain (Wang et al., 1996).

The role of *porc*, *zw3* and *dsh* in Wg signaling was originally recognized following examination of the segmentation phenotypes of embryos that lack both maternal and zygotic activities. These mutations were identified as the result of systematic screening of zygotic lethal mutations in germline mosaics (Perrimon et al., 1989). We have recently identified several new segment polarity mutants using this approach (Perrimon et al., 1996; and unpublished), and in this study we describe the identification and characterization of one of them that we have named *sugarless* (*sgl*). We show that the *sgl* mutation impairs signaling by Wg, and encodes a homologue of vertebrate UDP-glucose dehydrogenase, an enzyme essential for the biosynthesis of proteoglycans (PGs). We find that overexpression of Wg can bypass the requirement for *sgl*. Our results are consistent with a model suggesting that PGs facilitate the interaction of Wg with its receptor *Dfz2* by regulating the diffusion of secreted Wg.

MATERIALS AND METHODS

Genetics of *sgl*

A single *P*[*lacZ*, *ry*⁺] element insertion *l(3)08310* (Spradling et al., 1995), located at 65D04-05, was identified in a screen for maternal effects of zygotic lethal mutations (Perrimon et al., 1996) that was associated with a fully penetrant maternal effect phenotype. The association of lethality with this insertion was confirmed in two ways. First, *sgl* mutants do not complement the deficient line *Df(3L)W5.4* (Anderson et al., 1995) that covers the region from 65A to 65E. Second, we mobilized the P element associated with the *sgl* mutation using the *y w*; $\Delta 2-3$, *Sb/TM6* strain (Robertson et al., 1988). Out of 108 excision lines, 67 were homozygous viable.

Maternal effect phenotype of *sgl*

Females with germline clones were generated using the autosomal 'FLP-DFS' technique (Chou and Perrimon, 1996). Briefly, virgin females of the genotype *sgl FRT^{2A}/TM3*, *Sb* were mated with males of the genotype *y w FLP²²/+*, *FRT^{2A} P[ovo^{D1}]/TM3*, *Sb*. The resulting progeny were heat-shocked at 37°C for 2 hours at the larval stages, and *y w FLP²²/+*; *sgl FRT^{2A}/P[ovo^{D1}]/FRT^{2A}* females carrying *sgl* homozygous germline clones were selected.

Introduction of a paternal wild-type copy rescues the *sgl* maternal effect phenotype partially (Perrimon et al., 1996). While *l(3)08310*, null embryos exhibit a severe segment polarity phenotype, approximately 30% of the paternally rescued embryos exhibit weak segment polarity phenotypes (partial fusion of denticle bands) and do not hatch.

Antibody staining and in situ hybridization

Fixation of embryos and antibody staining procedures were performed as described (Patel, 1994). Anti-Wg serum was a gift from S. Cumberledge and used at 1:500 dilution. Anti-En mAb4D9 was used at 1:300 dilution and obtained from Developmental Studies Hybridoma Bank (Patel et al., 1989). Antibody against the Crumbs protein was used at 1:50 dilution and obtained from E. Knust (Tepass and Knust, 1993).

In situ hybridization of whole-mount embryos was done with PCR-generated digoxigenin-labeled DNA probe (Lehmann and Tautz, 1994). *wg* digoxigenin-labeled DNA probe was prepared from a *wg* cDNA subcloned in the *pSP65* plasmid.

Molecular biology

Genomic DNAs flanking the *sgl* P-element insertion were obtained by plasmid rescue in *E. coli* (Cooley et al., 1988). To isolate *sgl* cDNAs, we screened a 0- to 4-hour embryonic cDNA library (Brown and Kafatos, 1988) with genomic DNA fragments obtained from plasmid rescue. Several 2.3 kb *sgl* cDNAs encompassing the entire coding region were isolated. DNAs were sequenced by Taq-polymerase cycle sequencing and an automatic sequencer. To define the P-element genomic insertion site, the rescued plasmid from *sgl* flies was also sequenced using a primer derived from the P-element.

Northern blots of total RNA or poly(A)⁺ RNA were carried out by standard procedures (Sambrook et al., 1989). Probes used were as follows: 1.87 kb *Sst*I-*Xho*I fragment of the *sgl* cDNA (see Fig. 3A for sites) and 0.6 kb *Hind*III-*Eco*RI DNA fragment of ribosomal gene *rp49* (O'Connell and Roshbash, 1984). Sequence alignments were produced using 'DNA star' software.

RNA injection rescue

To mark embryos for RNA injection rescues, the *sgl* mutant chromosome was recombined with a *trachealess* (*trh*) mutant (Wilk et al., 1996; Isaac and Andrew, 1996) located at 61 C1-2. *trh* mutants exhibit defective Filzkörper, which are easily scorable cuticle markers. Females carrying *trh sgl* homozygous germline clones were generated and mated with *sgl/trh* transheterozygous males. 50% of the embryos resulting from this cross are homozygous for the *sgl* mutation and exhibit strong segment polarity phenotypes, while the other 50% are paternally rescued and easily identifiable by their defective Filzkörper.

RNAs were produced by in vitro transcription (Sambrook et al., 1989) from full-length cDNA plasmids containing an SP6 promoter using cap analog GpppG (Stratagene). Transcribed RNA was resuspended in DEPC-treated water at a concentration of 0.2 µg/µl. Embryos generated from the cross above were collected and microinjected as described (Anderson and Nüsslein-Volhard, 1984). Injected embryos were allowed to complete development for 2 days at 18°C prior to preparation. Scoring of cuticles was as described (Wieschaus and Nüsslein-Volhard, 1986). Of 800 injected embryos derived from females with *trh sgl* germline clones, 168 *sgl* mutant embryos (*trh sgl/sgl*) developed scorable cuticle structures, and 30% of them were rescued.

Misexpression experiments

The *pairedGAL4/TM3* (*prdGAL4*) line used in this study is described in Yoffe et al. (1995). *prdGAL4* was recombined with the *sgl FRT^{2A}* chromosome to a *sgl prdGAL4 FRT^{2A}* chromosome that was used to generate homozygous germline clones. The *UASwg^{ts}* (*M7-2.1*) line is located on the third chromosome and was described previously (Wilder and Perrimon, 1995; Yoffe et al., 1995). This insertion is homozygous viable. The *UASwg^{ts}* was recombined with the *sgl* mutation to generate *sgl UASwg^{ts}*. *UAS wg* was a gift of Henry Krause (unpublished). *UAS arm^{s10}* was obtained from Mark Peifer (Pai et al., 1997). The insertion used is homozygous viable and located on the second chromosome. *UAShh* was obtained from P. Ingham (Ingham and Fietz, 1995).

RESULTS

sgl is a novel segment polarity gene

The mutation in *sgl* was originally identified in a large screen to characterize the maternal effects of zygotic lethal mutations (see Perrimon et al., 1996; and Materials and methods for details). Homozygous *sgl* mutant animals derived from heterozygous mothers die at the third instar larval or early pupal stages. In contrast, homozygous *sgl* mutant embryos derived from females lacking germline *sgl* activity (referred to as *sgl* null embryos throughout the text) die with segmentation defects that resemble the phenotypes of mutants in segment polarity genes (Fig. 1B). The *sgl* maternal effect is partially paternally rescuable (see Perrimon et al., 1996; and Materials and methods for details) indicating that the gene is expressed at least during both oogenesis and early embryonic development.

The cuticle phenotype of *sgl* is reminiscent of the phenotypes exhibited by mutations in either *wg* or *hedgehog* (*hh*), suggesting that it may be involved in either or both of these signaling pathways. To further determine the involvement of *sgl* in Wg or Hh signaling, we examined the expression of *wg* mRNA, Wg protein and En protein in *sgl* null mutant embryos. In the ventral embryonic epidermis, Wg signaling is required for maintenance of *en* transcription at stage 10 (DiNardo et al., 1988; Yoffe et al., 1995). Subsequently, En, through a signaling pathway mediated by Hh (Ingham et al., 1991; Lee et al., 1992), is required for the maintenance of *wg* transcription (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991). As shown in Fig. 1, *wg* mRNAs fade in *sgl* (Fig. 1E) mutants at stage 9. Similarly, *en* expression is affected, since En protein disappears from the epidermis by stage 10 (Fig. 1F). These observations are reminiscent of other segment polarity mutants (DiNardo et al., 1988; van den Heuvel et al., 1993; Yoffe et al., 1995; Manoukian et al., 1995; Alcado et al., 1996) and suggest a role for *sgl* in either Wg and/or Hh signaling. Double stainings for both En and Wg protein indicate that when En protein begins to fade during late stage 9 in *sgl* mutants, Wg protein has almost completely decayed (Fig. 1H). *wg* mRNA disappearing from the epidermis significantly earlier than En has also been observed in *porc* mutants and interpreted as being characteristic for genes acting upstream of *wg*. This observation is consistent with a role for *sgl* in an upstream part of the Wg signal transduction pathway.

sgl encodes UDP-glucose dehydrogenase

To identify the transcript associated with the *sgl* mutation, a 2.9 kb fragment of genomic DNA flanking the P-element insertion was cloned following P-element rescue. This fragment was subsequently used to screen a *Drosophila* genomic DNA library in order to obtain a continuous stretch of genomic DNA representing the region of the P-element insertion. A screen for cDNAs, using genomic DNA fragments encompassing approximately 5 kb on both sides of the P-element insertion, identified a single cDNA of 2.3 kb mapping in the immediate vicinity of the P-

element insertion point. The length of the cDNA corresponds well with the length of a single signal obtained in a northern blot analysis using the cDNA as a probe (data not shown).

Several lines of evidence suggest that the isolated cDNA identifies the gene responsible for the *sgl* mutant phenotype. First, the P-element was found to be inserted into the 5'-untranslated leader region of the putative transcript 106 bp upstream of the putative ATG start codon (Fig. 2A). Second, northern blot analysis showed a complete loss of *sgl* maternal transcripts in 0-1.5 hour embryos derived from females with *sgl* germline clones, indicating that the P-element insertion disrupts the *sgl* transcript (Fig. 2B). Third, RNA transcribed in vitro using the isolated cDNA as a template was shown to be able to rescue the *sgl* mutant phenotype when injected into mutant embryos at the syncytial blastoderm stage (Fig. 3B,C).

Analysis of the nucleotide sequence of the *sgl* cDNA revealed a continuous open reading frame of 477-amino-acid residues (Fig. 2C). Comparison of the putative protein sequence derived from this reading frame with sequences represented in the databases revealed striking homology with bovine UDP-glucose dehydrogenase (Fig. 2C). The overall identity between Sgl and bovine UDP-glucose dehydrogenase is 67.7%. This enzyme provides the only pathway in all animals to convert UDP-glucose into UDP-glucuronic acid, an essential substrate for the biosynthesis of glycosaminoglycans (GAG) such as chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate (HS) (Hempel et

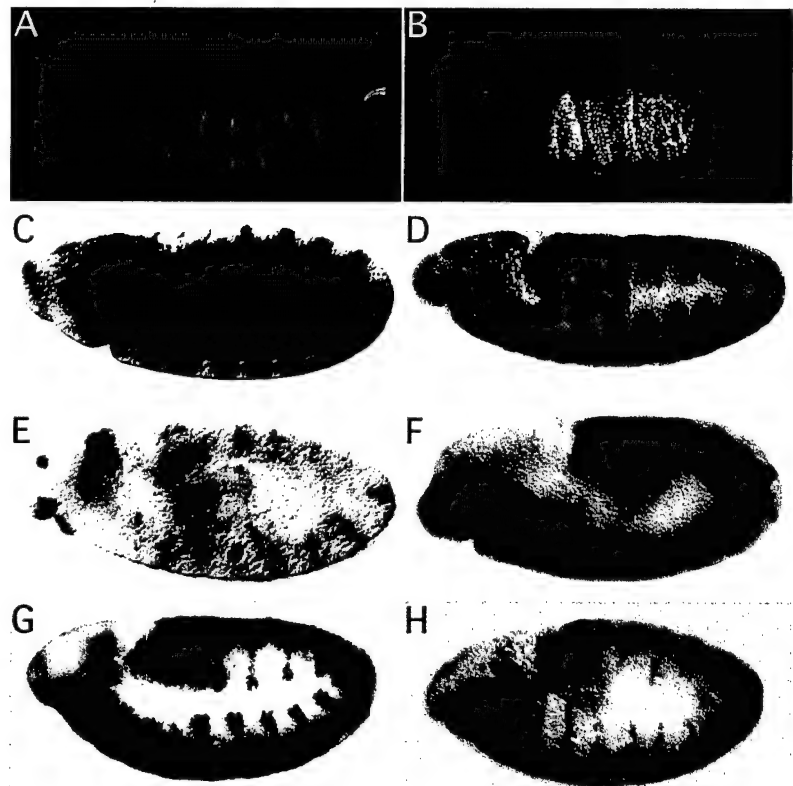
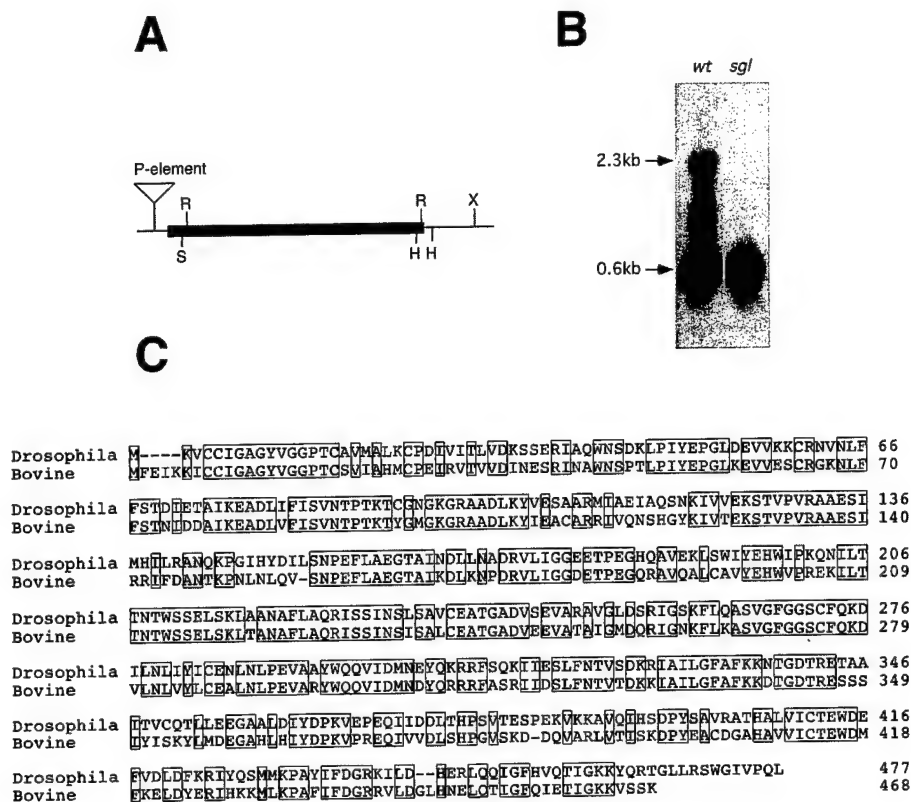


Fig. 1. Segment polarity phenotype associated with the *sgl* mutation. The cuticle phenotypes of wild type (A) and *sgl* (B) null embryos are shown; Transcription of *wg* mRNA is shown at stage 9 in wild type (C) and *sgl* (E) embryos; the expression of En protein is shown at stage 10 in wild type (D) and *sgl* (F) null embryos. The expression pattern of both Wg (blue/black) and En protein (brown) is shown at stage 10 in wild type (G) and at late stage-9 *sgl* (H) null embryos.

Fig. 2. Molecular characterization of *sgl* and northern blot analysis. (A) Restriction map of the *sgl* cDNA. The insertion of the P-element *l(3)08310* is indicated and corresponds to nucleotide 113 of the untranslated region of the *sgl* cDNA (106 bp upstream of the putative ATG start codon). The position of the open reading frame encoding the Sgl protein is indicated by the heavy line. H, *Hind* III; R, *Eco*RI; S, *Sst*I; X, *Xho*I. (B) Northern blot analysis of *sgl* on RNA from 0–1.5 hour wild-type embryos and embryos derived from females with *sgl* germline clones. About 2 μ g of poly(A)⁺ RNA were loaded. The blot was probed with ³²P-labeled 1.87 kb *Sst*I–*Xho*I fragment of the *sgl* cDNA (see Fig. 2A for sites) and a probe specific for ribosomal protein gene r49, which served as loading control. The 2.3 kb mRNA corresponds to the *sgl* transcript and the 0.6 kb mRNA to the ribosomal protein gene 49 transcript. (C) Putative amino acid sequence of Sgl (*Drosophila*) and comparison of the amino acid sequences of Sgl protein (*Drosophila*) with Bovine UDP-glucose dehydrogenase (Bovine). Identical residues are boxed.



al., 1994). Consistent with its role in the biosynthesis of GAGs, *sgl* is expressed both maternally and ubiquitously throughout embryonic development (data not shown).

Sgl activity is required for Wg signaling in various tissues

The molecular nature of Sgl predicts that the biosynthesis of PGs is disrupted in *sgl* mutants (see Discussion for details). Since HS/heparin has been demonstrated to be important in Wg signaling in tissue culture experiments (Reichsman et al., 1996), it is anticipated that Wg signaling is affected in *sgl* mutants and that the cuticle phenotype seen in *sgl* mutants reflects a role of HS/heparin, and possibly other PGs, in Wg signaling. Studying the requirement of PGs for Wg signaling in the epidermis is complicated because of the codependence of Wg and Hh expression (Ingham et al., 1991; Lee et al., 1992). Thus, to determine the dependence of Wg signaling on the presence of intact PGs, we examined the phenotype associated with the *sgl* mutation in other tissues in which Wg is required for pattern formation but where its role can be distinguished from that of Hh.

One of these tissues is the anlage of the stomatogastric nervous system (SNS) (González-Gaitán and Jäckle, 1995). The SNS arises during stage 10 from a distinctive region of the invaginating foregut. During stage 11, three invaginations form in the dorsal epithelium of the foregut (Fig. 4A). Later these invaginations will form vesicles that migrate dorsally towards the brain to form the SNS. In *wg* mutant embryos only one of the three invaginations forms (Fig. 4B). Virtually identical phenotypes, i.e. only one invagination, are observed in embryos mutant for genes that have previously been shown to be involved

in Wg signaling like *dsh* and *arm* (González-Gaitán and Jäckle, 1995). In contrast, *zw3* mutant embryos form additional invaginations, which is consistent with the opposite phenotypes of *wg* and *zw3* mutants in the ventral cuticle (Siegfried et al., 1992). Altogether, the Wg signaling pathway in the ventral epidermis and in the development of the SNS appears to involve the same set of genes (González-Gaitán and Jäckle, 1995). In contrast to *wg* mutants, *hh* mutants, like wild-type embryos, form three SNS invaginations. Although *hh* is expressed in the SNS anlage, we could not detect any defects specifically associated with the SNS in *hh* mutants (data not shown). For this reason the stomatogastric nervous system provides a system with which to determine whether Sgl activity is required for Wg signaling.

In order to examine the role of *sgl* in the development of the SNS anlage, *sgl* null mutant embryos were stained with an antibody against the Crumbs protein, which labels the apical surfaces of epithelial cells throughout the embryo (Tepass and Knust, 1993). In contrast to the phenotypes seen in genes that have previously been implicated in transducing the Wg signal, we did not observe the one-invagination phenotype in *sgl* mutant embryos. Instead, 2–3 invaginations that are fused at the base are usually detected (Fig. 4C). This phenotype is reminiscent of a weak *wg* mutant phenotype, as observed in a weak *arm* allele (see Fig. 8I in González-Gaitán and Jäckle, 1995). However, in contrast to the ventral epidermis, where the phenotype of *sgl* resembles an amorphic *wg* phenotype, defects in the SNS are less severe, suggesting that Wg signaling in the SNS is not completely abolished. Interestingly, in contrast to the ventral epidermis of *sgl* mutant embryos, where Wg expression has disappeared at stage 10, Wg expression in the SNS anlage persists beyond this stage (Fig. 4E, also see Fig.

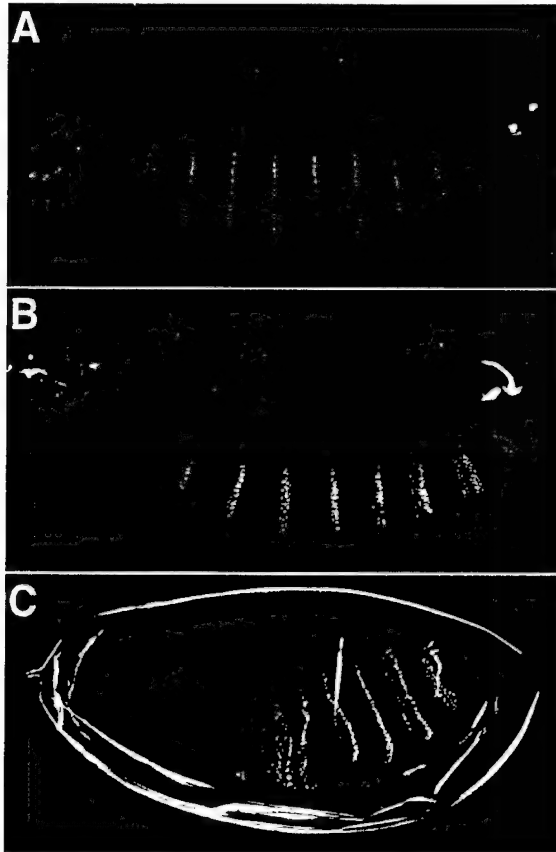


Fig. 3. Rescue of the *sgl* maternal effect phenotype by RNA injection. (A) Cuticle phenotype of a paternally rescued *sgl* embryo marked with a *trh* mutation (see Materials and methods for details). Note the defective Filzkörper. (B) and (C) are two examples of *sgl* null embryos derived from germline clones injected with *sgl* RNAs transcribed from the 2.3 kb *sgl* cDNA. Note that (B) is a fully rescued embryo in which the ventral denticle bands as well as head and tail structures are fully restored; (C) shows a partially rescued embryo.

1E). This observation suggests that the difference in severity of phenotypes between the SNS and the ventral epidermis may originate from the different modes of Wg regulation deployed in the respective tissues.

We have also examined the effect of loss of Sgl activity on the formation of the Malpighian tubules and the midgut constrictions. Both tissues require Wg activity for their development. In *wg* mutants only two short Malpighian tubules form instead of four (Skaer and Martinez-Arias, 1992) and the second constriction of the midgut does not form (Bienz, 1994). *sgl* null embryos develop four very short Malpighian tubules, a phenotype that would be expected to result from a partial loss of *wg* activity (data not shown). In addition, as observed in *wg* mutant embryos, *sgl* null embryos do not form the second midgut constriction. This is likely due to loss of expression of the homeobox gene *Ultrabithorax* (*ubx*), which is dependent on Wg signaling (Bienz, 1994) (Fig. 4G).

In summary, the observation of *wg*-like phenotypes in at least four tissues that depend on Wg inputs for their development further substantiates the requirement of *sgl* activity in Wg signaling. Based upon the severity of the mutant phenotypes,

it is apparent that in some tissues the requirement for Sgl is not absolute.

Ectopic expression of Wg can bypass the requirement for Sgl

Studies in the SNS and the Malpighian tubules suggest that Sgl is not absolutely essential in all tissues to implement the Wg signal, but instead may be involved in modulating the strength of the cellular response to Wg. To test this hypothesis we examined the effect of ectopic expression of Wg in the ventral epidermis of *sgl* null mutant embryos. We reasoned that if Sgl promotes the cellular response to the Wg signal, ectopic expression of various amounts of Wg should elicit dosage-dependent effects. We used the GAL4/UAS system of targeted gene expression (Brand and Perrimon, 1993) to misexpress various forms of Wg in *sgl* mutant embryos. A *prd*GAL4 line was used that drives the expression of a UAS-target gene in the *paired* (*prd*) pair-rule expression domain from stages 8 to 13 (Yoffe et al., 1995).

We first examined whether ectopic expression of Wg^{ts} effectively restores the naked cuticle as it does in *wg* and *porc* mutant embryos (Yoffe et al., 1995; Manoukian et al., 1995). In wild-type embryos, ectopic expression of Wg is associated with ectopic *en* as well as generation of naked cuticle (Yoffe et al.,

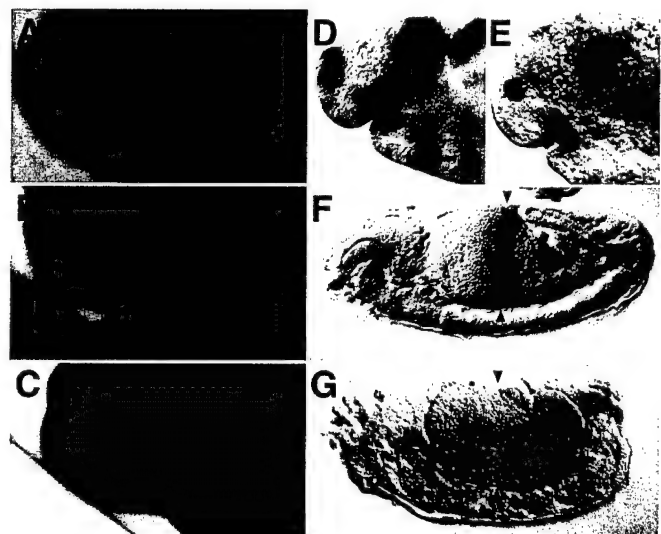


Fig. 4. Development of the stomatogastric nervous system (SNS) in *sgl* mutants. (A-C) Embryos are stained with anti-Crumbs antibody: wild type (A), *wg* (B) and *sgl* (C) null embryos are shown. The SNS is derived from three invaginations forming in the dorsal epithelium of the developing foregut of wild-type embryos at stage 10 (A). In a *wg* mutant embryo only one invagination is formed (B). In *sgl* (C) mutant embryos three invaginations are formed and fused at the base. This phenotype is reminiscent of *wg* hypomorphs and suggests that in *sgl* mutants Wg activity is reduced but not completely abolished. (D,E) Expression of Wg protein at stage 10 in wild type (D) and *sgl* (E) null embryos. In contrast to *wg* expression in the ventral epidermis, which is lost (see Fig. 1E,H), *wg* expression in the SNS anlage persists in *sgl* (E) mutants. (F,G) Expression of labial (*lab*) protein in a wild type (F) and a *sgl* (G) null embryo at stage 15. Labial staining, marking the position where the second midgut constriction will form in a wild-type embryo (F), is absent from a *sgl* (G) null embryo. Arrowheads mark the positions in which the constriction will form (F and G).

1995). As shown in Fig. 5, in *sgl* mutant embryos, expression of *prdGAL4/UASwg^{ts}* does not generate naked cuticle (Fig. 5A), while in control experiments, *prdGAL4/UASwg^{ts}* induced the expansion of En stripes in *sgl* zygotic mutant embryos with maternal supply (data not shown), as was shown previously (Yoffe et al., 1995). This result demonstrates that *sgl* is required for implementation of the Wg signal.

In light of the requirement of Sgl activity for Wg signaling during SNS formation, we reasoned that the inability of Wg^{ts} to generate naked cuticle in *sgl* mutant embryos may be due to the low activity of Wg^{ts} under these experimental conditions. These experiments were conducted at 16°C, which is the permissive temperature for Wg^{ts} (van den Heuvel et al., 1993; Wilder and Perrimon, 1995). At this temperature, GAL4 is not as efficient as at higher temperatures (Brand et al., 1994), and the activity of Wg^{ts} may be relatively low. Thus, we repeated the same misexpression experiment in the embryonic epidermis using a *UAS-wild type wg* construct. In wild-type embryos, expression of wild-type Wg protein in the paired expression domain results in deletion of denticle bands when embryos are allowed to develop at either 25°C (Fig. 5B) or 16°C (Fig. 5C). Wild-type Wg protein has stronger activity than Wg^{ts}, since expression of Wg^{ts} under identical conditions only causes the deletion of one row of denticles (Yoffe et al., 1995). Strikingly, at 25°C, expression of *prdGAL4/UASwg* in *sgl* mutant embryos induces naked cuticle (Fig. 5D), demonstrating that Wg signaling can occur in the absence of *sgl* activity. However, we observed only a weak effect of ectopic Wg, indicated by the formation of narrow regions of naked cuticle when the same experiment was conducted at 16°C (Fig. 5E). These results indicate that ectopic expression of Wg can rescue the defects of cuticle patterning in *sgl* in a dose-dependent manner.

We also examined the effect of misexpression of Hh in the paired domain in *sgl* null mutant embryos. As shown in Fig. 5F, *prdGAL4/UAShh* can effectively induce naked cuticle in *sgl* null mutant embryos at 16°C (not shown) or 25°C. Strikingly, in embryos mutant for *sgl*, Wg protein is maintained/induced in the paired domain where ectopic Hh is expressed at stage 11 (Fig. 5G). This result is consistent with the Wg misexpression experiments since the effect of Hh is mediated by wg.

To further determine at what level Sgl is required for Wg signaling, we examined the epistatic relationships between Sgl and Arm by misexpressing a gain of function Arm protein (Arm^{s10}) in which 54 N-terminal amino acid residues of Arm are deleted (Pai et al., 1997). As shown in Fig. 5H, expression of this constitutively active, Wg-independent form of Arm under the control of *prdGAL4*, in

sgl null embryos leads to the formation of naked cuticle in the paired domain. Restoration of naked cuticle by expression of constitutively active Arm confirms that it is in fact loss of Wg signaling that leads to the *sgl* mutant phenotype, and that furthermore, *arm* is epistatic to *sgl*-activity. The formation of naked cuticle is most likely the result of ectopic expression of wg, triggered by ectopic *en* and *hh*, which occurs in response to ectopic expression of Arm^{s10} (Pai et al., 1997).

DISCUSSION

Sgl is involved in Wg signaling

We have identified and characterized a novel segment polarity gene, *sgl*, which encodes an enzyme involved in proteoglycan (PG) biosynthesis. Embryos that develop in the absence of both maternal and zygotic *sgl* gene products exhibit cuticle defects and defects in gene expression in the ventral epidermis that are identical to those observed in *wg* or *hh* mutant embryos (Fig. 1). To demonstrate that normal expression of *sgl* is required for proper Wg signaling, we analyzed the requirement for *sgl* in

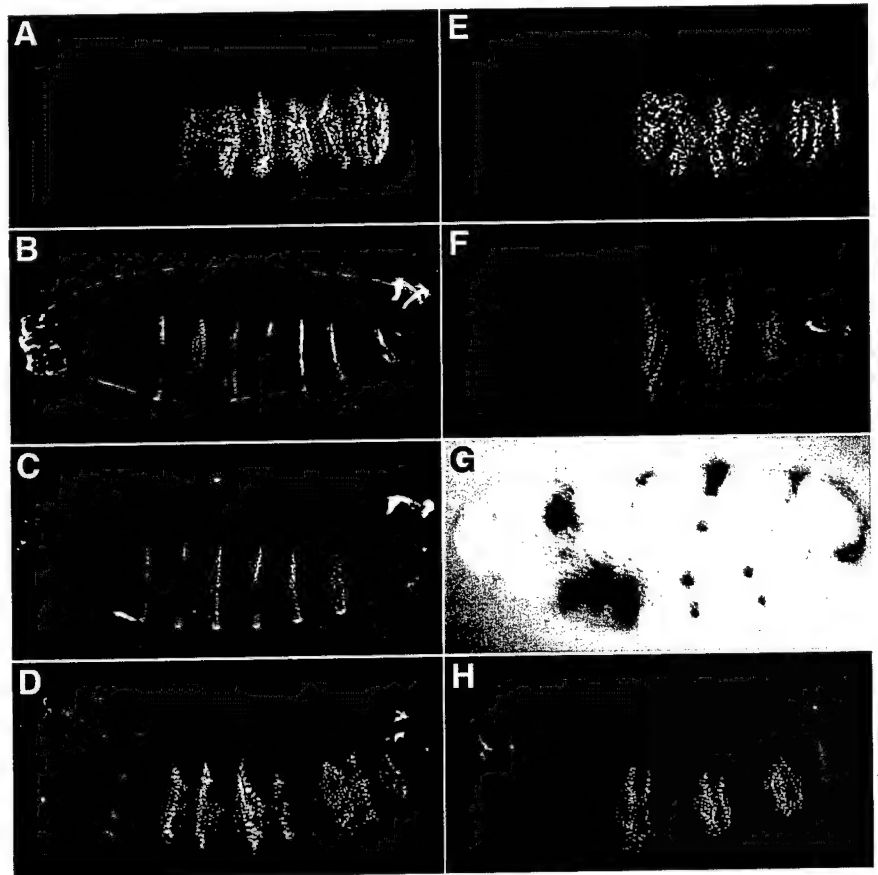


Fig. 5. Misexpression of *wg^{ts}*, *wg*, *hh* and *arm^{s10}* in *sgl* mutants. Except for (B) and (C), which are controls of *UASwg/prdGAL4* in wild-type backgrounds, all panels show embryos derived from females with *sgl* germline clones. The cuticle phenotypes shown are: *sgl prdGAL4/sgl UASwg^{ts}* (A), *UASwg/+; prdGAL4/+* (B,C), *UASwg/+; sgl prdGAL4/sgl* (D,E), *UAShh/+; sgl prdGAL4/sgl* (F). *UASarm^{s10}/+; sgl prdGAL4/sgl* (H). Embryos shown in (A) and (E) developed at 16°C. All the others developed at 25°C. (G) Wg protein expression at stage 11 in a *UAShh/+; sgl,prdGAL4/sgl* embryo. Note that while stripes of endogenous Wg protein fade completely, new stripes of Wg protein are induced by ectopic expression of Hh.

SNS formation, where *wg* expression does not depend on Hh activity (Fig. 4). Interestingly, we found that Wg signaling is only partially blocked in *sgl* null mutant embryos during SNS formation. We have also examined the effect of loss of Sgl activity on the formation of the Malpighian tubules and the midgut constrictions, two tissues that require Wg activity for their development (Fig. 4). In *sgl* mutants, the second midgut constriction does not form, as is observed in *wg* mutants, and four short Malpighian tubules are present, a phenotype reminiscent to a partial loss of *wg* function. Altogether, these observations suggest that the requirement for Sgl activity in Wg signaling is tissue specific, since implementation of the Wg signal can occur to some extent in some tissues in the total absence of Sgl. Our misexpression studies of *wg* in *sgl* mutants are consistent with this view because we demonstrate that high levels of ectopic Wg can bypass the requirement for Sgl activity.

Our study illustrates the critical requirement for PGs during embryogenesis, and we demonstrate that at least the Wg signaling pathway is affected in *sgl* mutants. However, it is likely that other signaling pathways that involve PGs are perturbed in the absence of Sgl activity. *sgl* mutant embryos do not exhibit cuticle phenotypes reminiscent to loss of cadherins or EGF receptor tyrosine kinase or TGF- β signaling pathways; however, preliminary results indicate that FGF signaling is affected in *sgl* mutants (X. Lin, A. Michelson and N. Perrimon, unpublished data). Finally, the requirement for Sgl in Hh signaling remains to be determined. We demonstrate that misexpression of Hh can effectively induce/maintain ectopic Wg and induce naked cuticle in *sgl* mutants, supporting a notion that Sgl is not involved in Hh signaling. Nevertheless, since we have not tested whether misexpression of a weaker Hh protein could signal in a *sgl* mutant background, there is a formal possibility for a dose-dependent requirement of *sgl* in Hh signaling, as observed for Wg.

In conclusion, based upon examination of the *sgl* mutant phenotypes, we propose that PGs are required for efficient Wg signaling. Their requirement appears to be specific to particular signaling pathways, since we do not observe a general disruption of the cellular context but instead disruption of specific signaling activities such as Wg.

***sgl* encodes an enzyme involved in proteoglycan biosynthesis**

PGs are ubiquitous macromolecules associated with the cell surface and the ECM of a wide range of cells that play central roles in morphogenesis, neurite outgrowth, angiogenesis and tissue repairs (reviewed by Bernfield et al., 1992; David, 1993; Kjellén and Lindahl, 1991; Yanagishita and Hascall, 1992). The biological function of PGs is mostly attributed to the highly negatively charged glycosaminoglycan (GAG) chains that interact with positively charged side chains of proteins.

GAGs are polymers of disaccharide repeats, which are often highly sulfated and negatively charged. Except for the nonsulfated polysaccharide hyaluronan, which occurs in the form of free GAG chains, all other GAGs identified, including heparin, heparan sulfate (HS), chondroitin sulfate and keratan sulfate, are covalently bound to a protein core to form a PG (reviewed by Kjellén and Lindahl, 1991). With the exception of keratan sulfate, all GAGs require D-glucuronic acid as a substrate for biosynthesis of their polysaccharide chains. The molecular analysis of *sgl* reveals that it encodes a homologue of bovine UDP-glucose dehydrogenase, which converts UDP-D-glucose to UDP-D-glu-

cronic acid. We therefore predict that except for keratan sulfate, biosynthesis of all other GAGs is disrupted in *sgl* null mutant embryos. Consistent with its role in biosynthesis of many proteoglycan polysaccharides, *sgl* is expressed both maternally and ubiquitously throughout embryonic development.

We propose that, in the absence of Sgl protein, Wg signaling is perturbed due to lack of or abnormal biosynthesis of PGs. If the general synthesis of PGs is perturbed in *sgl* mutants, then it is expected that other signaling pathways, which utilize PGs as coreceptor(s), may also be disrupted in the absence of Sgl proteins. Consistent with this hypothesis, we have observed that mesoderm migration in *sgl* mutants is defective (X. Lin, A. Michelson and N. Perrimon, unpublished) and similar to embryos carrying mutations in *heartless*, a *Drosophila* homologue of the FGF receptor (Beiman et al., 1996; Gisselbrecht et al., 1996). The availability of *sgl* mutants will allow us to examine the roles of PGs in signaling mediated by other growth factors, including FGFs.

Proteoglycans and Wnt signaling

Biochemically, there is ample evidence for the importance of PGs in Wnt-1/Wg signaling. It has been demonstrated that both mouse Wnt-1 and Wg are associated with the cell surface (Papkoff and Schryver, 1990; van den Heuvel et al., 1993; Reichsman et al., 1996) and the ECM (Bradley and Brown, 1990; Gonzalez et al., 1991; van den Heuvel et al., 1993; Reichsman et al., 1996). The association of Wnt-1/Wg with the cell surface or the ECM can be released by addition of exogenous HS or heparin, and heparin can directly bind to purified Wnt-1 and Wg protein (Bradley and Brown, 1990; Reichsman et al., 1996), suggesting that the association of Wnt-1/Wg proteins with the cell surface or the ECM is likely due to the direct interaction of Wnt-1/Wg protein with HSPGs. Further, recent tissue culture experiments have shown that HSPGs can modulate both extracellular localization of the Wg protein and Wg signaling (Reichsman et al., 1996). Treatment of Wg-responsive cells with heparinase, or blocking HS sulfation with perchlorate, results in the reduction of Wg activity. Consistent with these results, our studies demonstrate that loss of a component required for biosynthesis of PGs results in defects in Wg signaling and establishes a critical role for PGs in signal transduction *in vivo*.

In addition to Wnt-1/Wg protein, other Wnt family proteins have also been shown to be associated with the cell surface and to be effectively released into culture medium by the highly negatively charged polyanions heparin or suramin (Burrus and McMahon, 1995). Given the fact that the Wnt family of proteins are highly conserved, it is reasonable to suggest that PGs are also likely to be required for signaling by other, if not all, Wnt family members. In support of this view, recent experiments demonstrate that PGs, most likely HSPGs, are required for maintenance of Wnt-11 expression in the ureter tips (Kispert et al., 1996).

A model for the role of PGs in Wg signaling

Among the growth factors that interact with PGs, members of the FGF family have been particularly well studied. It has been proposed that HSPGs are required for dimerization of FGF receptors (Spivak-Kroizmon et al., 1994; Schlessinger et al., 1995). In an alternative model, HSPGs have been proposed to reduce the dimensionality of ligand diffusion from three to two dimensions (Schlessinger et al., 1995). Our genetic experiments demonstrate that in *sgl* mutant embryos, overexpression

of Wg protein can bypass the requirement for PGs to transduce the Wg signal to receiving cells. Based on these results, we propose that the function of PGs is to increase the local concentration of Wg ligand for its receptor. Binding of Wg protein to PGs on the cell surface or the ECM reduces the diffusion of the Wg ligand so that the Wg molecules are more likely to bind to the less abundant Wg receptor. In the absence of PGs, the concentration of Wg protein presented on the cell surface may be lower than its threshold concentration, and the efficiency of Wg signaling will be reduced. Overexpression of ectopic Wg protein can compensate for the loss of Wg protein on the cell surface and therefore bypass the requirement for PGs.

This model is consistent with our observation that in the SNS and in the Malpighian tubules Wg signaling is not completely abolished in *sgl* mutants (Fig. 4). In the ventral epidermis, however, the situation appears to be different as the loss of *Sgl* activity mimics a complete loss of Wg activity (Fig. 1). This observation most likely reflects the fact that in the ventral epidermis, the maintenance of *wg* transcription requires Wg signaling itself, either directly in an autocrine pathway, or via the Hh-mediated feedback loop. The reduction in Wg efficiency caused by lack of PGs is amplified through the feedback loop and rapidly leads to a complete loss of *wg* transcription, resulting in the amorphic phenotype.

Growth factors and HSPGs

Accumulating evidence has demonstrated that coreceptors, such as HSPG, function as indispensable components for signaling by a number of growth factors (Schlessinger et al., 1995; Massagué, 1996). For example, FGFs require HSPGs as coreceptors for signaling via a tyrosine kinase receptor. TGF- β binds to a serine/threonine kinase receptor in association with the membrane protein betaglycan (Schlessinger et al., 1995; Massagué, 1996). Either GDNF or CNTF require GPI-anchored proteins to mediate signaling (Massagué, 1996; Stahl and Yancopoulos, 1993). Binding of growth factors to coreceptors such as cell surface HSPGs will not transmit a signal, but will modulate the ability of growth factors, or the signaling receptors, to generate a biological response.

While the function of these molecules has been proposed to limit ligand diffusion from the cell surface, and to initiate dimerization of receptors, we speculate that these molecules may mediate other functional aspects of growth factors. For instance, Wg protein functions both as a short-range inducer in the ventral epidermis (van den Heuvel et al., 1989; DiNardo et al., 1988; Vincent and Lawrence, 1994) and as a long-range organizer in imaginal discs (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1995). Recent evidence suggests that Wg can act directly and at long range as a gradient morphogen (Zecca et al., 1996). If HSPG is a major limiting factor for diffusion of Wg protein, one would expect that dynamic changes of the expression of HSPGs may regulate the diffusion of Wg and eventually control the signaling range of Wg protein. Interestingly, a Glypican-related HSPG, named Dally, was identified, and *dally* mutants show wing notching with loss of wing margin structures (Nakato et al., 1995), an effect seen in *wg* and *dsh* mutants (Couso et al., 1994), suggesting a potential involvement of Dally in Wg signaling. A *Drosophila* homologue of vertebrate Syndecans has also been characterized (Spring et al., 1994). Syndecan is a transmembrane HSPG and represents the major source of HSPGs in epithelial cells.

Syndecan has been demonstrated to function as a coreceptor for FGF signaling (Bernfield et al., 1992; David, 1993). Further genetic and biochemical studies should reveal whether Dally and/or Syndecan play a direct role in Wg signaling.

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Note

The DNA sequence of UDP-glucose dehydrogenase has been submitted to GenBank and has been assigned the accession number: AF009013.

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Note

The gene we call *sugarless* was characterized by Binari et al. and Härry et al., who named the gene *Kiwi* and *Suppenkasper*, respectively. Following discussion with Dr. M. Ashburner, it was agreed to refer to the gene as *sugarless* (*sgl*) in subsequent publications.

Role of heparan sulfate proteoglycans in cell–cell signaling in *Drosophila*

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Abstract

Heparan sulfate proteoglycans (HSPGs) are abundant molecules associated with the cell surface and extracellular matrix, and consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached. Although these molecules have been the focus of intense biochemical studies in vitro, their biological functions in vivo were unclear until recently. We have undertaken an in vivo functional study of HSPGs in *Drosophila*. Our studies, as well as others, demonstrate the critical roles of HSPGs in several major signaling pathways, including fibroblast growth factor (FGF), Wnt, Hedgehog (Hh) and TGF- β . Our results also suggest that specific HS GAG chain modifications, as well as specific HSPG protein cores, are involved in specific signaling pathways. © 2000 Elsevier Science B.V./International Society of Matrix Biology. All rights reserved.

Keywords: Heparan sulfate proteoglycans; *Drosophila*; Cell signaling

1. Introduction

Heparan sulfate proteoglycans (HSPGs) are cell surface macromolecules that consist of a protein core to which heparan sulfate (HS) glycosaminoglycan (GAG) chains are attached (Bernfield et al., 1999). Several families of HSPGs can be classified according to the structure of the protein cores (Fig. 1). In addition to the diverse nature of the core proteins, enormous structural heterogeneity can be generated through specific HS chain modifications during their biosynthesis (Fig. 2). Thus, both the nature of the core proteins and modifications of HS-GAG chains can determine the specificity and function of HSPGs. A large body of evidence, mainly from in vitro biochemical studies, have implicated that HSPGs are important in a variety of cellular functions such as

cell adhesion, motility, proliferation, differentiation and morphogenesis (Iozzo, 1998; Bernfield et al., 1999). In the context of signal transduction, HSPGs have been proposed to act as co-receptors for a number of growth factors, internalization of receptors and transport of signaling molecules. However, the function of HSPGs in vivo has been unclear. Recent genetic studies in both *Drosophila* and mice have begun to uncover the functions of HSPGs in vivo in specific signaling pathways involved in cell differentiation and morphogenesis. This review summarizes the work from our laboratory on the in vivo functional studies of HSPGs in *Drosophila*. For a more extensive review, see Perrimon and Bernfield (2000).

2. Identification and isolation of mutations involved in the biosynthesis of HS GAGs

The development of the *Drosophila* embryo requires several key-signaling molecules for its pattern-

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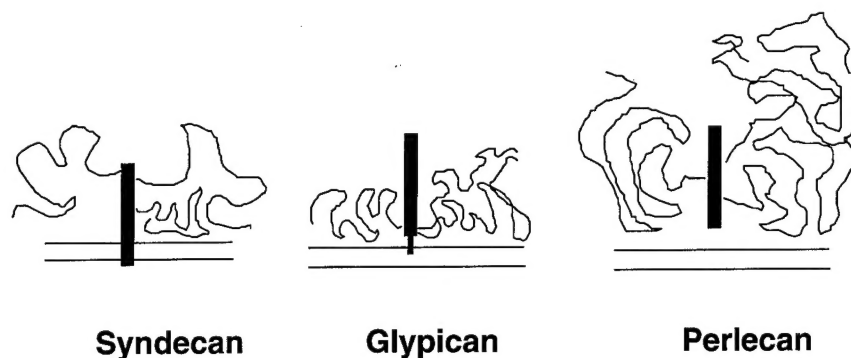


Fig. 1. Structures of HSPGs associated with cell surfaces. The syndecan and glypican family members are two major cell surface HSPGs, both of which are integral membrane proteins. The syndecan core proteins are transmembrane proteins that contain a highly conserved short (34–38 residue) carboxy-terminal cytoplasmic domain. The HS chains on the syndecans are linked to serine residues that are distal from the plasma membrane. The glypican core proteins are disulfide-stabilized globular core proteins linked to the plasma membrane by a GPI linkage. The HS chains on the glypicans are linked to serine residues adjacent to the plasma membrane. Perlecan are secreted HSPG bearing HS chains linked to amino termini of the core protein. In mammalian tissues, perlecan is present in nearly all basement membranes as well as cartilage.

ing, particularly in Wingless (Wg), a member of Wnt family, and Hedgehog (Hh). Maintenance of the normal segmentation pattern of the developing embryo is the result of reciprocal interactions between cells producing and cells receiving the Wg protein in the adjacent segments (Siegfried and Perrimon, 1994). Segmentation can be clearly visualized in the cuticle of the hatched larva as an alternating pattern of

‘naked’ cuticle and cuticle covered with small hairs called denticles. Wg signaling is required for specification of the ‘naked’ cuticle. Loss-of-function mutations involved in Wg signaling lead to a cuticle pattern with the absence of naked cuticle. Through a feed back mechanism, Hh is required for maintenance of Wg production. Mutations in Hh signaling result in a loss of *wg* expression and thus, lead to

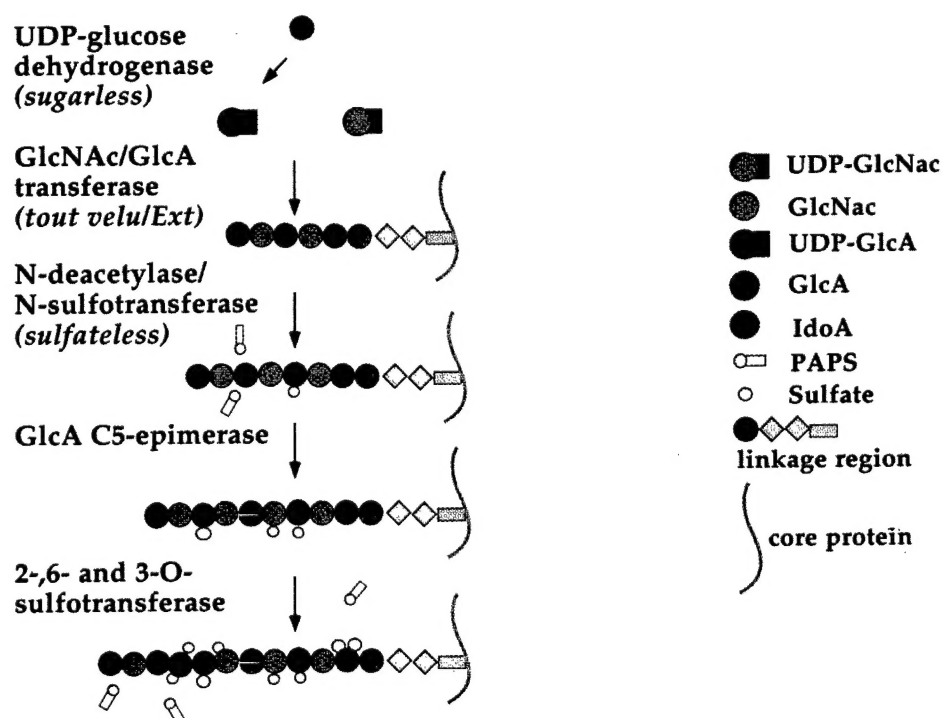


Fig. 2. HS chain biosynthesis (adapted from Perrimon and Bernfield, 2000). The chains are synthesized on the core protein by the sequential action of individual glycosyltransferases. A common tetrasaccharide linkage region is formed, followed by the addition of alternating GlcA and GlcNAc residues, producing in turn the precursor chain. This chain is then enzymatically modified by deacetylation and *N*-sulfation, epimerization and *O*-sulfation, yielding individual chains whose sequence is distinct from all the other chains.

similar cuticle defects as in *wg* mutants. Mutants with *wg*- or *hh*-like cuticle phenotypes are classified as segment polarity genes (Siegfried and Perrimon, 1994).

In a genetic screen to characterize the maternal effects of zygotic lethal mutations (Perrimon et al., 1996), we have identified several segment polarity genes including *sugarless* (*sgl*) (Binari et al., 1997; Haecker et al., 1997; Haerry et al., 1997), *sulfateless* (*sfl*) (Lin and Perrimon, 1999) and *tout velu* (*ttv*) (Bellaiche et al., 1998; The et al., 1999). In the absence of both maternal and zygotic expression of these genes, embryos die with a segment polarity phenotype. The cuticle phenotypes of these mutants are reminiscent of the phenotypes exhibited by mutations in either *wg* or *hh*, suggesting that these genes are involved in either or both of these signaling pathways. Molecular characterization of these genes revealed that they encode enzymes involved in the biosynthesis of HS GAGs. As illustrated in Fig. 2, *Sgl* encodes a homolog of UDP-D-glucose dehydrogenase (Hempel et al., 1994), which produces UDP-D-glucuronic acid that is a substrate for the biosynthesis of HS GAGs. *Sfl* is a homolog of heparan sulfate *N*-deacetylase/*N*-sulfotransferase (Hashimoto et al., 1992) that is required for the modification of HS GAG. Molecular characterization of *ttv* revealed that it encodes a member of the *Ext* gene family implicated in the human multiple exostoses (Ext) syndrome (Stickens et al., 1996). Both tissue culture experiments (McCormick et al., 1998) and biochemical studies (Lind et al., 1998) suggest that Ext proteins function as HS polymerases. The cuticle phenotypes associated with *sgl*, *sfl* and *ttv* as segment polarity genes suggest that HSPGs play key roles in Wg and/or Hh signaling.

3. HSPGs are required for Wg signaling

The *wg*-like cuticle phenotypes associated with mutations involved in the biosynthesis of HS GAGs implicate that HSPGs are required for Wg signaling. To further demonstrate a role of HSPGs in Wg signaling, we examined the development of several other embryonic tissues that require Wg activity. These include the development of the stomatogastric nervous system (SNS) and the second mid-gut constriction. Examinations of both *sgl* and *sfl* null embryos revealed that both the development of the SNS and the mid-gut constriction are abnormal, and resemble those found in *wg* mutants (Haecker et al., 1997). Altogether, our results implicate a requirement for HSPGs activity in Wg signaling during embryonic development.

HSPGs are also required for Wg signaling in the development of *Drosophila* imaginal discs. Wg is re-

quired for dorso/ventral (D/V) patterning and acts as a short-range inducer to activate the expression of several genes such as *neuralized* (*neu*) at the wing margin. Wg also functions as a morphogen to directly activate the transcription of several target genes, including *distalless* (*dll*), in a concentration-dependent manner. In *sfl* mutant wing discs, the expression of *neu* is abolished and Dll expression is also markedly reduced. These results indicate a role for HSPGs in both short-range and long-range Wg effects during wing-disc development (Lin and Perrimon, 1999).

To explore the mechanism(s) of how HSPGs participate in Wg signaling, we have ectopically expressed Wg protein in *sgl* or *sfl* null embryos using the UAS-Gal4 technique. Over expression of Wg protein can partially rescue Wg signaling in a dose-dependent manner in *sgl* and *sfl* null embryos (Haecker et al., 1997). These results suggest that HSPGs function in Wg signaling by limiting its diffusion and thereby facilitating the binding of Wg to its receptor (Haecker et al., 1997).

Consistent with our genetic evidence for a role of HSPG in Wg signaling, experiments in tissue culture demonstrated that Wg protein can directly bind to heparin. Wg signaling can be inhibited by removal of the heparan sulfate with heparinase or by treatment of cells with sodium perchlorate, a competitive inhibitor that blocks the sulfation of proteoglycans (Reichsman et al., 1996). Altogether, the results from both in vivo and in vitro experiments strongly argue that HSPGs play a key role in Wg signaling.

4. In vivo evidence for HSPGs function in the FGF signaling pathways

A large body of biochemical and cellular evidence shows that HSPGs are an essential component for signaling mediated by the FGF protein family (Ornitz, 2000). However, there has been no direct demonstration that HSPGs are required for the biological activity of FGFs in a developmental system in vivo. The availability of HS-GAG biosynthesis mutants allowed us to examine the in vivo role of HSPGs in FGF signaling. Two FGF receptors, Heartless (Htl) and Breathless (Btl) have been identified in *Drosophila*. Htl and Btl are required for the migration of mesodermal and tracheal cells respectively. In both *sgl* and *sfl* null mutants, the migration of mesodermal and tracheal cells is defective, phenotypes reminiscent of *htl* and *btl* null mutants (Lin et al., 1999). We have further demonstrated that both Htl- and Btl-dependent MAPK activation is significantly reduced in the embryos lacking *sfl* and *sgl* activities. Furthermore, a constitutively activated form of Htl partially rescues the mesodermal cell migration defects in *sfl* and *sgl*

mutants. These results provide the first *in vivo* evidence that HSPGs are essential for FGF signaling.

5. Specific function of Ttv in the regulation of Hh movement and signaling

Ttv was initially identified as a segment polarity gene (Perrimon et al., 1996). Molecular characterization of *ttv* revealed that it encodes a member of the *Ext* gene family that functions as HS polymerases. Further analysis of *ttv* reveals that it plays a key role in the movement of Hh through a field of cells (Bellaiche et al., 1998). These studies suggested that the membrane-targeted cholesterol-modified Hh molecule requires HSPGs to be either trapped by receiving cells or move from cells to cells (The et al., 1999). Interestingly, when other signal transduction pathways were examined, it was found that in the absence of *ttv* activity only Hh signaling, but not Wg or FGF signaling, is affected (The et al., 1999). These results argue that biosynthesis of HS GAG can contribute to the specificity of HS GAG in signaling. One model to explain the specificity of Ttv in Hh action is that Hh signaling is more sensitive to a reduction in HSPGs concentration than Wg and FGF signaling. Alternatively, an Hh-specific HSPG may exist and Ttv may be responsible for its synthesis (The et al., 1999). Ttv is not the only example suggesting that specific modification of HS GAG can determine the specificity of proteoglycans in signaling. Other evidence comes from the analysis of *Drosophila pipe* mutant that encodes a putative HS 2-OST (Sen et al., 1998). *Pipe* is expressed in the ventral part of the egg chamber and has been proposed to activate the serine protease cascade that leads to production of the active Toll ligand Spätzle. Another example of specific developmental defects associated with a mutation in a HS 2-OST has been found in mice (Bullock et al., 1998).

6. Role of the HSPG core proteins in signal transduction

In *Drosophila*, a single *syndecan* gene, two *glypican* genes, *dally* and *dally-like* (Baeg et al., unpublished), and a *perlecan* homolog have been identified. However, detailed phenotypic analyses are only available for *dally*. Dally was initially identified as a mutant affecting cell division patterning in the developing central nervous system (Nakato et al., 1995). A detailed analysis of *dally* has implicated Dally in Wg signaling (Lin and Perrimon, 1999; Tsuda et al., 1999). Loss of *dally* activity, both in the embryo and imaginal discs, generates phenotypes reminiscent of loss of

Wg activity. Genetic interaction experiments are consistent with a model in which Dally acts as a co-receptor for the Wg transducing receptor encoded by the seven transmembrane protein Frizzled 2 (Lin and Perrimon, 1999; Tsuda et al., 1999). Interestingly, *dally* expression is developmentally regulated and is co-expressed with Frizzled 2 in the embryo (Lin and Perrimon, 1999), suggesting that, as previously observed for Frizzled 2 (Cadigan et al., 1998), the level of the HSPG co-receptor is tightly regulated for proper Wg signaling.

Dally has also been found to act in Decapentaplegic (Dpp, a member of the TGF β family) signaling (Jackson et al., 1997). A reduction in *dpp* levels enhances the defects associated with *dally* mutations in the eye, antenna, and genitalia. Furthermore, additional copies of *dpp* rescue the defects in these tissues. These genetic interactions have led to the hypothesis that Dally regulates Dpp activity (Jackson et al., 1997). However, the activity of Dally in Dpp signaling is limited only to imaginal discs since no defects associated with Dpp signaling during embryonic development have been found in *dally* mutant. Taken together, these results suggest that Dally is developmentally regulated and interacts with only a subset of signaling pathways.

7. Perspectives

Genetic analyses of enzymes involved in HS chain biosynthesis and the core proteins have demonstrated critical roles of HSPGs in developmental processes. It is now apparent that HSPGs are implicated in several major signal transduction pathways, and that both the modification of HS biosynthesis and their specific protein cores can contribute to the regulation of specific pathways. It is now important to address how specific modifications and protein cores regulate cell–cell signaling. Furthermore, several signaling molecules including Wg, Hh and Dpp can function as morphogens and we need to better understand the contribution of HSPGs in establishing the protein gradients of these signaling molecules. Further analyses of the mutant phenotypes associated with the remaining enzymes and core proteins will lead to a better understanding of these issues.

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